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07/01/89

EP0417160

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(5) International Patent Classification 4 :</b> <b>C12Q 1/68, C07H 21/04,</b> <b>// C07K 7/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 89/11547</b> <b>(43) International Publication Date:</b> 30 November 1989 (30.11.89)
<b>(21) International Application Number:</b> <b>PCT/US89/02169</b> <b>(22) International Filing Date:</b> 18 May 1989 (18.05.89)  <b>(30) Priority data:</b> 196,660 20 May 1988 (20.05.88) US 258,212 14 October 1988 (14.10.88) US 347,506 4 May 1989 (04.05.89) US  <b>(71) Applicant:</b> CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US).  <b>(72) Inventors:</b> ERLICH, Henry, A. ; 3936 Rhoda Avenue, Oakland, CA 94602 (US). HORN, Glenn, T. ; 1400 Worcester, Apt. 7607, Framingham, MA 10701 (US). BUGAWAN, Teodorica, L. ; 15524 Faris Street, San Leandro, CA 94579 (US).	<b>(74) Agent:</b> HALLUIN, Albert, P.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> METHOD FOR HLA DP TYPING  <b>(57) Abstract</b> <p>A process for determining the genotype of an individual with respect to the alleles at the HLA DP locus involves obtaining a sample of nucleic acid from the individual and hybridizing the nucleic acids with a panel of probes specific for variant segments of DPalpha and DPbeta genes. Because the variation between DPbeta alleles is highly dispersed throughout the second exon of the DPbeta gene, the discovery of many different DPbeta alleles makes the process far more discriminating and informative than cellular, RFLP, or serological methods. The process can also be carried out on amplified nucleic acid produced by the polymerase chain reaction using primers specific for the second exon of the DPalpha and DPbeta genes. HLA DP DNA typing methods are useful in the prevention of graft rejection and host versus graft disease, in determining susceptibility to autoimmune diseases, in providing evidence concerning the derivation from an individual of forensic samples, and in paternity testing.</p>		

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## METHOD FOR HLA DP TYPING

This invention relates to a method and compositions for determining the HLA DP genotype of an individual. In a preferred embodiment, the invention relates to using gene amplification methodology disclosed and claimed in U.S. Patent Nos. 4,683,195 and 4,683,202 and the dot-blot and allele-specific oligonucleotide probe technology as disclosed and claimed in U.S. Patent 4,683,194. The methods and probes of the invention specifically relate to the detection of the polymorphic class II HLA DP genes. The invention relates to the fields of molecular biology, diagnostic medicine, and forensics.

The class II loci of the human major histocompatibility complex encode the HLA D cell surface glycoproteins which are expressed on B lymphocytes, activated T lymphocytes, macrophages, and dendritic cells. These proteins, which are individually designated DR, DQ, and DP, are composed of an alpha and a highly polymorphic beta subunit and are responsible for the presentation of antigen to T cells. The variability in the highly polymorphic beta subunit is localized to the amino-terminal extracellular domain, which is thought to interact with the T cell receptor and antigen peptide fragments. The genes encoding the class II HLA proteins are located on the short of chromosome six in humans. The genes encoding the HLA DPalpha and DPbeta chains are clustered at the centromeric end of this region and, perhaps due to low levels of expression, were the last of the HLA D genes to be discovered and are, therefore, the least well characterized. The structures, sequences, and polymorphisms in the HLA D region have been reviewed in Trowsdale *et al.*, 1985, *Immunol. Rev.* 85:5-43, incorporated herein by reference.

The polymorphism of the HLA D region gene products has, in general, been defined by serologic typing reagents and by the mixed lymphocyte culture (MLC) reaction in which T cell proliferation in response to homozygous typing cells (HTC) is measured in culture. The HLA DP antigens were originally defined by their ability to stimulate a strong secondary response in specifically primed T cells, a method known as primed lymphocyte typing (PLT) and described by Mawas *et al.*, 1981, *Tissue Antigens* 15:458-466; Want *et al.*, 1978, *Immunogenetics* 6:107-115; and Shaw *et al.*, 1980, *J. Exp. Med.* 152:565-580. The HLA DP antigens elicit only a weak response in a primary MLC, and unlike the studies of HLA DR and HLA DQ polymorphism, the analysis of allelic variation in the HLA DP region has been complicated by the lack of availability of serologic reagents and of typing

cells. In addition, the specific cell lines used in the PLT assay are difficult to generate, and the typing assay is slow and somewhat variable from laboratory to laboratory.

Cellular (see Odum *et al.*, 1987, Tissue Antigens 29:101-109), biochemical (see Lotteau *et al.*, 1987, Immunogenetics 25:403-407), and restriction fragment length polymorphism (RFLP, see Hyldig-Nielsen *et al.*, 1987, Proc. Natl. Acad. Sci. USA 84:1644-1648) analyses have indicated that the degree of polymorphism in the DP region may be more extensive than the currently serologically, immunologically, or PLT-defined DPw1 through DPw6 types. The RFLP approach showed that the RFLP of the DP region was relatively extensive and that some of the fragments were strongly associated with certain DP antigens. However, the RFLP technique has certain limitations. An allele carrying a variant sequence is identifiable only if the variant nucleotide is within the recognition site of a restriction enzyme used in the analysis or if a polymorphic restriction site is in linkage disequilibrium with a specific coding sequence variation. In addition, RFLP analysis simply provides evidence that a coding sequence variation exists but does not provide information on the exact nature of the variation. Moreover, relatively large fragments of the genomic nucleic acid must be used for the analysis. This latter requirement often rules out the use of samples which have been kept under conditions which result in the degradation of the genomic DNA.

Accurate DP typing may prove important in several medical applications. Genetic recombination between the DP loci and the serologically typed DR loci can occur such that serologically identical sibs may not be matched at DP. The HLA DP differences have been revealed by RFLP analysis in several cases of acute graft vs. host disease between apparently HLA-identical donor and recipient pairs, as described by Amar *et al.*, 1987, J. Immunology 138:1947-1953. Furthermore, several autoimmune diseases, including coeliac disease, have been shown to be associated with specific DP types, defined either by PLT, Odum *et al.*, 1986, Tissue Antigens 28:245-250, or by RFLP, Howell *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:222-226, analysis.

A significant improvement in DNA amplification, the polymerase chain reaction (PCR) technique, was disclosed by Mullis in U.S. Patent No. 4,683,195, and methods for utilizing PCR in the cloning and detection of nucleic acids were disclosed by Mullis *et al.* in U.S. Patent No. 4,683,202, both of which patent disclosures are hereby incorporated herein by reference. In the PCR technique, short oligonucleotide primers are prepared to match opposite ends of the sequence to be amplified. The sequence between the primers need not be known. A sample of nucleic acid (DNA or RNA, although RNA is first

converted to cDNA in the PCR process) is extracted and denatured, preferably by heat, and hybridized with oligonucleotide primers which are present in molar excess. Polymerization is catalyzed by a polymerase in the presence of deoxynucleotide triphosphates (dNTPs). This results in two "long products" which contain the respective primers at their 5'-termini, covalently linked to the newly synthesized complements of the original strands. The replicated DNA is again denatured, hybridized with oligonucleotide primers, returned to polymerizing conditions, and a second cycle of replication is initiated. The second cycle provides the two original strands, the two long products from cycle 1 and two long products of cycle 2, and two "short products" replicated from the long products produced in cycle 1. The short products contain sequences (sense or antisense) derived from the target sequence and flanked at the 5' end with a primer and at the 3'-end with a sequence complementary to a primer. On each additional cycle, the number of short products is replicated exponentially. Thus, the PCR process causes the amplification of a specific target sequence and allows for the detection of sequences initially present in a sample in only extremely small amounts.

Allelic sequence variations in the gene encoding beta-globin of hemoglobin and in the gene encoding HLA DQalpha have been detected by utilizing allele-specific oligonucleotides (ASO), which will only anneal to sequences that match them perfectly, as described by Saiki *et al.*, 1986, *Nature* 324:163-166. These studies also utilized the PCR procedure to amplify the DNA sequences present in the samples and a dot-blot technique to detect probe hybridization to the sample.

The present invention solves the above listed problems in HLA DP typing and provides a relatively rapid, convenient, practical, accurate, and reproducible method for determining HLA DP genotypes. The method is based in part upon the finding that there are a large number of previously unreported DPbeta alleles. The variation between alleles is of a highly dispersed nature. The present invention also provides nucleic acid probes for the regions of the HLA DPbeta gene that are most informative in discriminating between different DP alleles for use in the method.

These oligonucleotide probes are called SSOs (sequence specific oligonucleotides), which, under the appropriate conditions, are able to bind specifically to their complementary sequences. If a particular probe can be used to identify uniquely an allele, the probe is called an allele specific oligonucleotide (ASO). Because of the dispersed nature of the variation between the DPbeta alleles, rarely is any one probe able to identify uniquely a specific DPbeta allele. Rather, according to the methods of the present

invention, the identity of an allele is inferred from the pattern of binding of a panel of probes, with each individual probe of the panel specific for different segments of the HLA DP gene. The probes comprise a nucleotide sequence totally complementary to a variant sequence of a variable segment of a DP gene. The complementary sequence of the probes is usually from 10 to 30 nucleotides in length, most often 17 to 19 nucleotides in length.

As noted above, PCR is quite an important tool in nucleic acid based diagnostic methods. The novel probes of the invention can of course be used to detect specific sequences in PCR-amplified DNA. To enable practice of PCR-based, HLA DP DNA typing, the present invention provides oligonucleotide primers that allow for the amplification of informative DP regions via PCR.

Accordingly, one aspect of the invention is a process for determining an individual's HLA DP genotype from a nucleic acid containing sample obtained from the individual comprising: (a) amplifying a target region of said nucleic acid which contains a polymorphic region of an HLA DP gene; (b) hybridizing the amplified nucleic acid with a panel of sequence specific oligonucleotide (SSO) probes specific for variant segments of HLA DP genes under conditions that allow said SSO probes and amplified nucleic acids to form stable hybrid duplexes; and (c) detecting hybrids formed between the amplified nucleic acids and the SSO probes.

Another aspect of the invention relates to kits useful for determining the HLA DP genotype of an individual; these kits comprise a panel of SSO probes for allelic variant sequences in said target region; and (b) instructions for determining the genotype by utilizing kit ingredients.

To aid in understanding the invention, several terms are defined below.

"Genotype" refers to a description of the genotypic variants of an allele present in an individual (or sample). The genotype of an individual may be determined, for example, by the use of serologic reagents, by the use of typing cells, by RFLP analysis of the genome, and, as discussed below, by the use of SSOs.

"HLA DP region" or "DP" refers to that region of the HLA D complex, described in Trowsdale *et al.*, *supra*, which lies on the centromeric side of the HLA D region and contains two alpha and two beta genes, one pair of which are pseudogenes.

"Oligonucleotide" refers to primers, probes, nucleic acid fragments to be detected, nucleic acid controls, and unlabeled blocking oligomers and is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides. The exact size of an oligonucleotide will depend upon many factors and the ultimate function or use of the

oligonucleotide. Oligonucleotides can be prepared by any suitable method. Such methods include, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphotriester method of Narang *et al.*, 1979, Meth. Enzymol. 68:90; the phosphodiester method of Brown *et al.*, 1979, Meth. Enzymol. 68:109; the diethylphosphoramidite method of Beaucage *et al.*, 1981, Tetrahedron Lett. 22:1859-1862; and the solid support method in U.S. Patent No. 4,458,066.

"Polymorphic" or "DNA polymorphism" refers to the condition in which two or more variations of a specific DNA sequence coexist in the same interbreeding population.

"Primer" refers to an oligonucleotide, whether natural or synthetic, capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, i.e., in the presence of four different nucleotide triphosphates and an agent for polymerization (i.e., DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably an oligodeoxyribonucleotide and is single stranded for maximum efficiency in amplification, but may also be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The exact length of a primer will depend on many factors, but typically ranges from 15 to 25 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template. An example of a non-complementary sequence which may be incorporated into the primer is a sequence which encodes a restriction enzyme recognition site (see U.S. Patent No. 4,800,159).

"Primer," as used herein, may refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding one or both ends of the target region to be amplified. For instance, if a nucleic acid sequence is inferred from a protein sequence, a "primer" is actually a collection of primer oligonucleotides containing sequences representing all possible codon variations based on the degeneracy of the genetic code. One of the primer oligonucleotides in this collection will be homologous with the end of the target sequence. Likewise, if a "conserved" region shows significant levels of polymorphism in a population, mixtures of primers can be prepared that will amplify adjacent sequences. A primer can be labeled, if desired, by incorporating a label detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include <sup>32</sup>P, fluorescent dyes, electron-dense reagents, enzymes (as

commonly used in ELISAS), biotin, or haptens or proteins for which antisera or monoclonal antibodies are available. A labels can also be used to "capture" the primer, so as to facilitate the immobilization of either the primer or amplified DNA on a solid support.

"Restriction endonucleases" and "restriction enzymes" refer to enzymes, usually of bacterial origin, that cut double-stranded DNA at or near a specific nucleotide sequence.

"Restriction fragment length polymorphism" or "RFLP" refers to the differences in DNA nucleotide sequences that are randomly distributed throughout the entire genome and that produce different restriction endonuclease patterns for different individuals upon digestion of genomic DNA.

"Sequence-specific oligonucleotide" or "SSO" refers to oligonucleotides that have an exactly complementary sequence to the sequence to be detected, typically sequences characteristic of a particular DP allele, which under "sequence-specific hybridization conditions will hybridize only to that exact complementary target sequence. The hybridization is under stringent conditions. Stringent hybridization conditions are known in the art, and are described, for example, in Maniatis *et al.*, Molecular Cloning: A Laboratory Manual (New York, Cold Spring Harbor Laboratory, 1982). Depending on the sequences being analyzed, one or more sequence-specific oligonucleotides may be employed for each sequence. The terms "probe" and "SSO probe" are used interchangeably with SSO.

"Target region" refers to a region of a nucleic acid which is to be analyzed, which usually contains polymorphic DNA sequences.

"Thermostable polymerase enzyme" refers to an enzyme which is relatively stable to heat and which catalyzes the polymerization of nucleotides to form primer extension products that are complementary to one of the nucleic acid strands of the target sequence. Generally, the enzyme will initiate synthesis at the 3'-end of the target sequence utilizing the primer and will proceed in the 5'-direction along the template until synthesis terminates. A purified thermostable polymerase enzyme is described more fully in European Patent Office (EPO) Publication 258,017, incorporated herein by reference, and is commercially available from Perkin-Elmer Cetus Instruments.

The present invention provides processes and reagents for determining the HLA DP genotype of an individual. In part, the invention results from the discovery of polymorphisms in the variable second exon of the HLA DPbeta genes by the combined methods of PCR amplification, cloning, and DNA sequencing. As a result, 22 DPbeta (DPB) allelic variants have been discovered. Based upon the novel sequence of these DP



genes, SSO probes are provided for the detection of the variant genotypes. The variations between the different DP alleles are dispersed. Therefore, one probe alone is rarely able to identify uniquely a specific DPbeta allele. Rather, the identity of an allele is inferred from the pattern of binding of a panel of probes, with each individual probe of the panel specific to different segments of a DP gene.

In a preferred embodiment of the invention, the process for DNA based typing of HLA DP genotypes is comprised of amplifying a nucleic acid sequence which contains a variable portion of an HLA DP gene, determining the variant HLA DP sequence present using SSO probes; and inferring the HLA DP genotype from the pattern of binding of the SSO probes to the amplified target sequence. To facilitate practice of this preferred embodiment, the present invention provides primers useful in amplifying by PCR the HLA DP target region.

In this preferred method, a sample containing nucleic acid is obtained from an individual whose HLA DP genotype is to be determined. Any type of tissue containing HLA DP nucleic acid may be used for purposes of the present invention. Because the present invention also is compatible with amplified nucleic acids, and because the PCR technique can amplify extremely small quantities of nucleic acid, samples containing vanishingly small amounts of nucleic acid can be typed for the presence of particular HLA DP variants by the method of the present invention. For instance, even a single hair, contains enough DNA for purposes of the present invention, as evidenced by the work with DQalpha described by Higuchi *et al.*, 1988, *Nature* 332:543-546.

In general, the nucleic acid in the sample will be DNA, most usually genomic DNA. However, the present invention can also be practiced with other nucleic acids, such as messenger RNA or cloned DNA, and the nucleic acid may be either single-stranded or double-stranded in the sample and still be suitable for purposes of the present invention. Those of skill in the art recognize that whatever the nature of the nucleic acid, the nucleic acid can be typed by the present method merely by taking appropriate steps at the relevant stage of the process. If PCR is used to amplify the nucleic acid in the sample, then the sample will usually comprise double-stranded DNA when typed with the novel probes of the invention.

As noted above, in a preferred embodiment, the HLA DP typing method and probes of the invention are used in conjunction with PCR-amplified target DNA. Those practicing the present invention should note, however, that amplification of HLA DP target sequences in a sample may be accomplished by any known method which provides sufficient

amplification so that the target sequence may be detected by nucleic acid hybridization to an SSO probe. Although the PCR process is well known in the art (see U.S. Patent Nos. 4,683,195 and 4,683,202) and although a variety of commercial vendors, such as Perkin-Elmer/Cetus instruments, sell PCR reagents and publish PCR protocols, some general PCR information is provided below for purposes of clarity and full understanding of the invention to those unfamiliar with the PCR process.

To amplify a target nucleic acid sequence in a sample by PCR, the sequence must be accessible to the components of the amplification system. In general, this accessibility is ensured by isolating the nucleic acids from the sample. A variety of techniques for extracting nucleic acids from biological samples are known in the art. For example, see those described in Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, (New York, Cold Spring Harbor Laboratory, 1982). Alternatively, if the sample is fairly readily disruptable, the nucleic acid need not be purified prior to amplification by the PCR technique, i.e., if the sample is comprised of cells, particularly peripheral blood lymphocytes or amniocytes, lysis and dispersion of the intracellular components may be accomplished merely by suspending the cells in hypotonic buffer.

Because the nucleic acid in the sample is first denatured (assuming the sample nucleic acid is double-stranded) to begin the PCR process, and because simply heating some samples results in the disruption of cells, isolation of nucleic acid from the sample can sometimes be accomplished in conjunction with strand separation. Strand separation can be accomplished by any suitable denaturing method, however, including physical, chemical, or enzymatic means. Typical heat denaturation involves temperatures ranging from about 80°C to 105°C for times ranging from about 1 to 10 minutes. Strand separation may also be induced by a helicase, an enzyme capable of exhibiting helicase activity. For example, the enzyme RecA has helicase activity in the presence of ATP. The reaction conditions suitable for strand separation by helicases are known in the art (see Kuhn Hoffman-Berling, 1978, CSH-Quantitative Biology 43:63 and Radding, 1982, Ann. Rev. Genetics 16:405-436).

As noted above strand separation may be accomplished in conjunction with the isolation of the sample nucleic acid or as a separate step. In addition, strand separation can be achieved simultaneously with the next step in the PCR process: annealing of primers and synthesis of primer extension products. In this embodiment of the PCR process, the temperature is very carefully controlled so that strand separation and primer annealing and extension occur in equilibrium. In this embodiment of the PCR process, the reaction is

catalyzed by a heat-stable polymerase and carried out at an elevated temperature. The temperature is one at which the enzyme is thermostable, and at which the nucleic acids are in an equilibrium of single and double strands, so that sufficient primer will anneal to template strands to allow a reasonable rate of polymerization. In the preferred embodiment of the PCR process, however, strand separation is achieved by heating the reaction to a sufficiently high temperature for an effective time to cause the denaturation of the duplex, but not to cause an irreversible denaturation of the polymerase (see EP No. 258,017).

No matter how strand separation is achieved, however, once the strands are separated, the next step in PCR involves hybridizing the separated strands with primers that flank the target sequence. The primers are then extended to form complementary copies of the target strands, and the cycle of denaturation, hybridization, and extension is repeated as many times as necessary to obtain the desired amount of amplified nucleic acid.

As noted above, the present invention provides PCR primers for HLA DP DNA amplification and typing. These primers are complementary to sequences in the conserved regions that flank the target sequences in the variant regions of the HLA DP loci. For purposes of the present invention, the preferred variant region of the HLA DP loci is the second exon of the DPalpha and DPbeta genes. For successful PCR amplification, the present primers are designed so that the position at which each primer hybridizes along a duplex sequence is such that an extension product synthesized from one primer, when it is separated from its template (complement), serves as a template for the extension of the other primer to yield an amplified segment of nucleic acid of defined length. Moreover, primers are provided that will bind preferentially to the HLA DP region under selective annealing conditions.

Template-dependent extension of primers in PCR is catalyzed by a polymerizing agent in the presence of adequate amounts of four deoxyribonucleotide triphosphates (dATP, dGTP, dCTP, and dTTP) in a reaction medium comprised of the appropriate salts, metal cations, and pH buffering system. Suitable polymerizing agents are enzymes known to catalyze template-dependent DNA synthesis. For example, if the template is RNA, a suitable polymerizing agent to convert the RNA into a complementary DNA (cDNA) sequence is reverse transcriptase (RT), such as avian myeloblastosis virus RT. Once the target for amplification is DNA, suitable polymerases include, for example, *E. coli* DNA polymerase I or its Klenow fragment, T<sub>4</sub> DNA polymerase, and Taq polymerase, a heat stable DNA polymerase isolated from *Thermus aquaticus* and commercially available from Perkin-Elmer/Cetus Instruments (PECI). The latter enzyme is widely used in the

amplification and sequencing of nucleic acids. The reaction conditions for using DNA polymerases are known in the art, and are described in, for example, the treatise Methods in Enzymology, and in Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, *supra*.

5 The PCR method can be performed in a step-wise fashion, where after each step new reagents are added, or in a fashion where all of the reagents are added simultaneously, or in a partial step-wise fashion, where fresh or different reagents are added after a given number of steps. For example, if strand separation is induced by heat, and the polymerase is heat-sensitive, then the polymerase will have to be added after every round of strand separation. However, if, for example, a helicase is used for denaturation, or if a  
10 thermostable polymerase is used for extension, then all of the reagents may be added initially, or, alternatively, if molar ratios of reagents are of consequence to the reaction, the reagents may be replenished periodically as they are depleted by the synthetic reaction.

Those skilled in the art will know that the PCR process is most usually carried out as an automated process with a thermostable enzyme. In this process, the reaction mixture is  
15 cycled through a denaturing region, a primer annealing region, and a reaction region. A machine specifically adapted for use with a thermostable enzyme is disclosed more completely in EP 236,069 and is commercially available from PECL.

One reason, as noted above, the PCR process is important in the method of the present invention is that the PCR process can be used to amplify the sample nucleic acid prior to HLA DP DNA typing. Another important use of PCR for purposes of the present  
20 invention, however is for determining the nucleotide sequence of previously undiscovered allelic variants which exist in the HLA DP region, so that probes for those variants can be constructed and used in the present method. In this use of the PCR process, polymorphic regions of the DPalpha and DPbeta genes are amplified, and the nucleotide sequences of  
25 these polymorphic target regions, for example, the second exon of the DPalpha and DPbeta genes, are determined. As illustrated below, it is also useful for the cells containing a particular variant to be typed by serological typing, mixed lymphocyte typing, or primed lymphocyte typing to correlate the nucleotide sequence of a particular variant with the DP type established by prior art methods.

30 Analysis of the nucleotide sequence of the target region of a DP variant allele can be readily performed by direct analysis of the PCR products. A preferred sequencing protocol is described by Innis *et al.*, 1988, Proc. Natl. Acad. Sci. 85:9436-9440, incorporated herein by reference. A process for direct sequence analysis of PCR amplified products is also described by Saiki *et al.*, 1988, Science 239:487-491. Alternatively, the amplified

target sequence may be cloned prior to sequence analysis, as described by Scharf *et al.*, 1986, Science 233:1076-1078.

As discussed in the Examples below, a panel of DPw typed cells, representing a large number of different haplotypes, have been analyzed by PCR and nucleotide sequencing of the second exon of the DPbeta gene. As a result of this effort and similar efforts using samples obtained from a variety of individuals suffering from autoimmune diseases, discussed in greater detail below, 22 different allelic variants in this locus were discovered. In general, the results demonstrated that specific DPbeta sequences correlate with the standard PLT-defined DPw1 through DPw6 specificities. The rare exceptions may reflect the difficulty of obtaining a standard and reproducible PLT DPw typing system, which difficulties highlight the advantages of the present method. In this regard, it is relevant that the cell line Cox, originally typed as DPw1, has recently been retyped as DPw3 and contains the DPB3 allele.

Other interesting and important correlations between the serologically defined DP types and DP variant nucleotide sequences have been discovered as a result of the advances provided by the present invention. For instance, the DPw4 type of the prior art can now be subdivided into two DPbeta subtypes (designated DPB4.1 and DPB4.2) by the method of the present invention. The DPB4.2 allele was found in two (APD and LBI) DPw4 homozygous typing cells (HTCs) known to have an unusual DPw4 typing. DPbeta subtypes (designated DPB2.1 and DPB2.2) for the DPw2 type were also discovered as a result of the present invention.

Yet another significant advance provided by the present invention relates to the discovery of a large number of variant DPbeta alleles in a variety of cells that type by the prior art methods as DP "blank." These variant alleles have been designated with DPB numbers of 7 and above. Further analysis of DPw blank haplotypes will probably lead to the identification and characterization of additional DPbeta alleles that can then be typed by the present method. DPw blank cells are those which fail to stimulate the existing PLT panels of T cell reagents. Unlike the serologic DQw blank specificity, which, as described by Horn *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:6012-6016, is encoded by a unique DQB sequence, the DPw blank specificity is heterogeneous. Because the haplotypic frequency of the DPw blank is estimated to be 40%, the present invention is especially important in that it for the first time enables the subtyping of this large DPw type.

The availability of DPbeta DNA sequences from DPw typed cells not only makes possible the subdivision of the serological DPw types but also makes possible the

identification of the linear epitopes defined by serologic and cellular typing reagents. For example, the antibody SP42 reacts with DPw2 and DPw4 cells. The only sequence unique to these two specificities is the GlyGlyProMet (GGPM) at positions 84 to 87, which are therefore believed to constitute the SP42 epitope. Likewise, the antibody SP3 reacts with DPw3 and DPw6 cells, as well as with some additional cells like the DPw blank lines Tok and Akiba. These lines bear the specificity Cp63 and contain the closely related DPbeta alleles DPB9 and DPB12. The only sequence unique to SP3 reactive cells is the acidic AspGluAsp (DED) sequence at positions 55 to 57. The SP3 epitope therefore seems to map to this region of the DPbeta chain.

As yet another example of how the novel DP sequences of the invention allow the mapping of serologically defined epitopes, the antibody DP11.1 reacts with DPw2 and DPw4 cells. Bodmer *et al.*, 1987, Proc. Acad. Sci. USA 84:1644-1648, showed with Western blot analysis that this antibody binds to the DPalpha chain. Given the pattern of DPbeta polymorphisms provided by the present invention, this observation suggests that the DP11.1 antibody binds to a conformational determinant formed by the DPalpha chain and DPbeta chains containing the GlyGlyProMet (GGPM) sequence at positions 84 to 87.

Polymorphic residues in the HLA DP region may also constitute epitopes recognized by T cell clones. Cells from clone 1666 react with DPw5 and some DPw2 cells. ABL (DPB2.2) is positive and WPV (DPB2.1) is negative, as disclosed at the Tenth International Histocompatibility Workshop. These C1666 cells may recognize the GluAlaGlu (EAE) residues at position 55 to 57, because this sequence is unique to the DPB2.2 and DPB5 alleles. A Leu (as opposed to Phe) residue at position 35 is also unique to C1666 reactive cells.

The same approach of correlating specific polymorphic residues with DPw specificities can be used to map the epitopes defined by PLT typing. For example, the only difference between the DPB2.1 and DPB4.2 alleles is the Glu to Lys change at position 69. Because cells containing these alleles are distinguished from each other in the PLT system, the charge of the polymorphic position 69 residue is believed to be involved in the epitope recognized by PLT typing cells. Similarly, the only differences between the DPB3 and DPB6 alleles is the Lys to Glu substitution at position 69, as well as Val to Met change at position 76.

As illustrated by the foregoing, the novel DPbeta sequences provided by the present invention not only provides the basis for a very useful DPbeta DNA typing method but provides much useful information in the interpretation of serological DPbeta typing results.

The present invention also enables one to perform HLA DNA typing not only on the DPbeta locus but also on the DPalpha locus as well. As illustrated below, at the present time, only two variant alleles are known to exist at the second exon of the DPalpha locus (see Trowsdale *et al.*, *supra*). The protein sequences encoded by the second exon of these two alleles (these alleles are designated DPA1 and DPA2) differ from each other by only three amino acids, and these differences do not appear to correlate with any specific PLT-defined type. Thus, the DPalpha variation is much less extensive than predicted by the RFLP variation detected with DPalpha probes (see Hyldig-Nielsen *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84:1644-1648), suggesting that most of the polymorphisms detected in the DPalpha gene by RFLP analysis are either in noncoding sequences or in a nearby DPbeta locus. The observed correlations between DPw types and DPalpha RFLP markers therefore probably reflects linkage disequilibrium between DPbeta sequence variation and the polymorphic restriction sites detected with the DPalpha probe, as does the association between DRalpha RFLP markers and DR specificities (see Stetler *et al.*, 1985, *Proc. Natl. Acad. Sci. USA* 82:8100-8104).

The DNA sequences of the DPalpha and DPbeta genes serve as a useful starting point in the design of the sequence specific oligonucleotide probes of the present invention. These probes are designed so that, under stringent hybridization conditions, the probes hybridize specifically only to exactly complementary sequences in variant segments of the DPalpha and DPbeta alleles. These SSO probes may be of any length which spans the variant sequences in a variant region and allows for sequence specific hybridization, but preferably the hybridizing region of the probe is short, in the range of 10 to 30 bases, and more preferably is about 17 to 19 bases in length. For immobilization, the probe may also contain long stretches of poly T which can be fixed to a solid support by irradiation.

The SSO probes of the invention are also designed to hybridize specifically with a particular variant segment of a DP allele and to have destabilizing mismatches with the other variant sequences known for the particular segment. Preferably, the probes are specific for variant DNA segments in the variable second exons of the DPalpha and DPbeta genes, and even more preferably, the probes are specific for DNA segments encoding the residues near positions 8-11, 36, 55-57, 65-69, 76, and 84-87 of the second exon. Oligonucleotide probes which have been designed to hybridize specifically to the second exons of the DPbeta and DPalpha alleles are described in more detail below and in the Examples.

The probes of the invention can be synthesized and labeled using the techniques described above in the discussion of PCR primers. For example, the probe may be labeled

at the 5'-end with  $^{32}\text{P}$  by incubating the probe with  $^{32}\text{P}$ -ATP and kinase. A suitable non-radioactive label for SSO probes is horseradish peroxidase (HRP). Methods for preparing and detecting probes containing this label are described in the Examples below. For additional information on the use of such labeled probes, see U.S. Patent No. 4,789,630; 5 Saiki *et al.*, 1988, *N. Eng. J. Med.* 319:537-541; and Bugawan *et al.*, 1988, *Bio/Technology* 6:943-947, incorporated herein by reference. Useful chromogens include red leuco dye and TMB.

The probes of the invention can be used to identify the allelic sequences present in a sample by determining which of the SSO probes bind to the HLA DP sequences present in 10 the sample. Suitable assay methods for purposes of the present invention to detect hybrids formed between SSO probes and nucleic acid sequences in a sample are known in the art. For example, the detection can be accomplished using a dot blot format, as described in the Examples. In the dot blot format, the unlabeled amplified sample is bound to a membrane, the membrane incubated with labeled probe under suitable hybridization conditions, 15 unhybridized probe removed by washing, and the filter monitored for the presence of bound probe. When multiple samples are analyzed with few probes, a preferred process requires high stringency hybridization and wash conditions, allowing only perfectly matched hybrids to exist.

An alternative method is a "reverse" dot blot format, in which the amplified 20 sequence contains a label. In this format, the unlabeled SSO probes are bound to the membrane and exposed to the labeled sample under appropriately stringent hybridization conditions. Unhybridized labeled sample is then removed by washing under suitably stringent conditions, and the filter is then monitored for the presence of bound sequences. In another version of the "reverse" dot blot format, the SSO probe is labeled, and the 25 sample nucleic acid is unlabeled. After hybridization and washing, the labeled probe, or a labeled fragment of the probe, is released from the membrane and detected to determine if a sequence in the sample hybridized to the labeled oligonucleotide. Release of label can be achieved by digestion with a restriction enzyme that recognizes a restriction site in the duplex hybrid. This procedure, known as oligomer restriction, is described more fully in 30 U.S. Patent No. 4,683,194 and corresponding EP Patent Publication 164,054, incorporated herein by reference.

Whatever the method for determining which DP SSO probes of the invention hybridize to DP sequences in a sample, the central feature of the DP DNA typing method involves the identification of the HLA DP alleles present in the sample by analyzing the



pattern of binding of a panel of SSO probes. Although single probes of the invention can certainly be used to provide useful information, the variation in the DPbeta alleles is dispersed in nature, so rarely is any one probe able to identify uniquely a specific DP variant. Rather, as shown in the Examples, the identity of an allele is inferred from the pattern of binding of a panel of SSO probes, which are specific to different segments of the DPalpha and DPbeta genes.

DNA typing of HLA DP alleles is useful for many different purposes. For example, DPbeta polymorphism is involved in tissue rejection of allografts. Bone marrow transplantation studies revealed that three apparently HLA-identical donor-recipient pairs who exhibited acute graft versus host disease and weak MLC reactivity were, by RFLP analysis, all different in the DP region, as described by Amar *et al.*, *J. Immunology* 138:1947. Thus, useful methods for preventing graft rejection will involve matching the HLA DP types of the donor and recipient by applying the present method of HLA DP DNA typing: inferring the HLA alleles present in donor and host from the pattern of hybridization of SSO probes to DPbeta and/or DPalpha nucleic acid sequences present in samples obtained from both donor and host.

Another important application for the present method of HLA DP DNA typing is in determining an individual's susceptibility to autoimmune diseases linked to certain HLA DP alleles. The association of particular DP alleles with an autoimmune disease can be discovered by using the present method to determine the frequency with which a given allele is present in individuals suffering from the autoimmune disease and comparing that frequency with the frequency determined for healthy individuals of a control group, as has been done with other HLA typing systems. For example, Howell *et al.*, 1988, *Proc. Natl. Acad. Sci. USA* 85:222-226, reported a significant association between coeliac disease (CD) and a pair of HLA DPalpha and HLA DPbeta RFLPs. Such studies often provide the additional benefit of the discovery of previously uncharacterized DP alleles, in both patient and control groups. For instance, the DPB13 through DPB19 alleles, the sequences of which represent an important aspect of the present invention, were discovered in the course of studies involving autoimmune disease patients and control groups.

In just such a fashion, an initial study of four CD patients who were typed by the method of the present invention revealed that the DPB4.2 allele confers CD susceptibility. A more extensive study, based on samples from Italian individuals, also showed that the calculated relative risk (RR) of CD for the DPB4.2 allele is 9.3 and that six of the eleven CD patients typed by the present method carried the DPB3 allele. About 78% of CD

patients examined by the present method carry either the DPB4.2 or the DPB3 alleles, so that the RR for the presence of either of these alleles is 13.5. In yet another study of U.S. individuals with CD, an increase in the DPB1 allele frequency, as well as an increase in the DPB4.2 allele frequency, was observed when compared to the frequency of these alleles in a control group. Such studies also revealed that specific combinations of DQbeta alleles (DQB2), DQalpha alleles (DQA4) and DPbeta alleles (DPB4.2 or DPB3) are associated with increased risk of CD susceptibility.

As shown from the foregoing, the present invention provides important new methods for determining the CD susceptibility of an individual, which methods comprise determining whether an individual carries a DP allele associated with CD susceptibility. As shown herein, such CD susceptibility-conferring alleles include the DPB4.2, DPB3, and DPB1 alleles, and as DPB4.1 is a relatively common allele, there is an increased frequency of the genotype DPB4.1/4.2 in CD susceptible individuals.

As with CD, the present invention provides similar advances with respect to other serious autoimmune diseases. For instance, studies that correlate allelic frequency with disease show that the frequency of specific DPbeta alleles appears to be higher in insulin dependent diabetes mellitus (IDDM) and myasthenia gravis (MG) patients than in otherwise HLA matched controls. For MG, the frequency of DP alleles that code for the sequence DEAV at the 3' end of the second exon, such as DPB3 and DPB5, is increased. In addition, the frequency of the DPB4.1 allele in MG patients (relative to healthy control individuals) is dramatically decreased, suggesting that DPB4.1 may confer protection from MG. For IDDM, the frequency of DPbeta alleles DPB2.1, DPB1, DPB4.1, and DPB13 is increased. Many of the IDDM patients studied that carried the DPB13 allele also carried the HLA alleles B18 and DR3.

The DP typing methods of the present invention have also provided important advances in determining whether an individual is susceptible to certain forms of arthritis. For instance, patients with classical rheumatoid factor positive adult rheumatoid arthritis (ARA) show an increased frequency of the DPB4.2 allele, although more studies must be completed to ensure that this increase is statistically significant. In addition, scientists have long known that the DPw2 serologic type is associated with susceptibility to pauciarticular juvenile rheumatoid arthritis (JRA). As noted above, the present invention has for the first time allowed the serologic DPw2 type to be subdivided into two DPB DNA types, and the disease-susceptibility typing methods of the present invention demonstrate that the known DPw2-associated susceptibility to JRA is more specifically attributable to the DPB2.1

allele. About 55% of the JRA patients examined were positive for DPB2.1, while only 16% of control individuals had this allele, giving a RR of 6.3 for JRA in individuals with the DPB2.1 allele. The association of JRA with the DPB2.1 allele is independent of linkage with previously defined HLA DP region markers, and the significance of this DPB2.1 association with JRA can be more readily appreciated when one considers that the DPB2.1 sequence differs from the DPB4.2 allele (apparently not associated with JRA susceptibility) by only one amino acid at position 69 of the B1 domain. It should be noted, however, that most JRA patients with the DPB2.1 allele also had a disease-associated DR marker such as DRw8, DR5, or DRw6.

Thus, the present invention also provides a method for detecting JRA susceptibility that comprises treating HLA DP genomic DNA of an individual, preferably after amplification, with oligonucleotide probes specific for the DPB2.1 allele and determining if hybridization has occurred. General practice of the invention will, however, utilize the full panel of DPbeta probes. As noted above, the present method will be useful in determining the DP association with a wide variety of autoimmune diseases, not just JRA. This fact can be more readily grasped by considering the Table below, in which the allelic frequency (number of alleles of the indicated DP type divided by total number of DP alleles present and then multiplied by 100) for the various DP alleles in a number of patient groups and a control group is shown. In the Table, MS indicates multiple sclerosis patients.

## HLA-DP ALLELIC FREQUENCIES IN SELECTED PATIENT GROUPS

	<u>Allele</u>	<u>Controls (n=50)</u>	<u>MG (n=23)</u>	<u>JRA (n=44)</u>	<u>MS (n=21)</u>	<u>ARA (n=23)</u>
	1	6%	9%	3%	2%	5%
	2.1	10%	6%	30%	12%	12%
5	2.2	0%	2%	0%	6%	0%
	3	6%	13%	11%	17%	11%
	4.1	56%	25%	40%	48%	41%
	4.2	10%	10%	6%	6%	22%
	5	2%	10%	0%	0%	2%
10	6	2%	0%	2%	0%	2%
	7	0%	0%	0%	2%	0%
	8	0%	6%	0%	0%	0%
	9	0%	6%	1%	0%	0%
	10	1%	6%	1%	0%	2%
15	11	3%	2%	1%	0%	0%
	12	0%	0%	0%	0%	0%
	13	1%	2%	1%	2%	0%
	14	2%	0%	0%	2%	2%
	15	0%	0%	2%	0%	2%
20	16	0%	0%	1%	2%	0%
	17	1%	2%	0%	0%	2%
	18	0%	0%	0%	0%	0%
	19	0%	0%	0%	0%	0%
	20	0%	2%	0%	0%	0%

25 n = number of individuals typed; 2n is the number of chromosomes tested; controls were individuals from Northern California.

30 In general, these studies of autoimmune disease suggest that the change of polymorphic residues at positions 55, 56, and 69 of the DPbeta chain are involved in genetic susceptibility to autoimmune disease. For instance, the change at position 55 from alanine to aspartate occurs in both the DPB2.1 and DPB4.2 alleles; however, in IDDM, which appears to be linked to DPB2.1, there is also a change in the amino acid at position 69. It should be noted, though, that is is the entire allele, and not simply an amino acid at one position in an allele, which appears to confer susceptibility.

The effect of the HLA DP alleles on autoimmune disease susceptibility reflects similar findings in the HLA DR and HLA DQ loci. For instance, Scharf *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:3504-3508, and Horn *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:6012-6016, report that polymorphic residues at position 57 of the DQbeta chain and position 70 of the DRbeta I chain are implicated in genetic susceptibility to the autoimmune diseases pemphigus vulgaris (PV) and IDDM.

As noted above, it is anticipated that as medical technology develops, more disease or disease-prone states, including Grave's disease, systemic lupus erythromatosis, and Sjögren's Syndrome, will become known to be associated with various DP alleles. The present invention provides methods for distinguishing such alleles from other alleles and so provides a means to identify individuals at high risk for an autoimmune disease. In a preferred embodiment, an individual whose susceptibility is to be determined is analyzed for HLA DP type first by using the PCR method to amplify the target region of the HLA DP locus. Then, SSO probes are hybridized to the amplified target region, and the particular DP allele present in the amplified DNA is determined from the pattern of binding of the SSO probes. Finally, one determines whether the allele present in the amplified DNA is an allele associated with the autoimmune disease.

The present method, however, is not limited to the field of medical science in ability to provide significant benefits. DNA typing methods also now play a significant role in the important area of individual identification, whether for solving crimes, as when the identity of a criminal or victim is established by linking an individual with evidence left at the scene of a crime, or for solving other issues of a non-criminal nature, as when biological material is used to determine the maternity or paternity of an individual.

Whatever the purpose for which the present invention is employed, the differences between various DP alleles is key to the success of the method. The most significant differences between DP alleles can be detected quite readily when the various amino acid sequences encoded by the alleles are aligned and examined. Such an alignment is shown below, where a dash indicates identity with the DPB4.1 allele (for the various DPbeta alleles and the DPbeta pseudogene, designated SXB) or with the DPA1 allele (for the DPalpha DPA2 allele and the DPalpha pseudogene, designated SXA). In this depiction, the numbered positions are for the mature peptide subunits, allele designations are at left, and representative cell sources are at right.

## DPalpha Alleles

DP41: DHVSTYAAFVQTHRPTGEFMFEDEMFYVDLKKETVWHLEFGQAFSFEAQGLANIAILNNNLNTLIQRSNHTQATN (LB)  
 DPA2: -----Q-----R-----A-----  
 (SXA): E-Q-S-Y-----E-Q-N-E-M-P-P-IHT-D-G-R-I-G-VMARKH---R-NG KQ-W--D

## DPbeta Alleles

DP4.1 NYLFQGRQECYAFNGTQRFLERYIYNREEFARFSDVGEFRAVTELGRAAEYWNQKDILEEKRAVPDRMCRHNYELGCPMTLQRR (HHK)  
 DP4.2 -----V-----DE-----  
 DPB2.1 -----V-----DE-----E-----  
 DPB2.2 -----LV-----E-----E-----  
 DPB8 -----V-----DE-----E-----V-----DEAV-----  
 DPB5 -----LV-----E-----E-----DEAV-----  
 DPB7 -----V-----DE-----E-----V-----DEAV-----  
 DPB3 VY-L-----V-----DED-----L-----V-----DEAV-----  
 DPB6 VY-L-----V-----DED-----L-----E-----DEAV-----  
 DPB11 VY-L-----Q-Y-----DED-----L-----R-----DEAV-----  
 DPB13 VY-L-----Y-----DED-----E-----I-----DEAV-----  
 DPB1 VY-----Y-----DED-----V-----V-----DEAV-----  
 DPB10 VH-L-----V-----DED-----E-----V-----DEAV-----  
 DPB9 VH-L-----V-----DED-----E-----V-----DEAV-----  
 DPB12 --VH-L-----G-----S-----DED-----E-----V-R-----DEAV-----  
 DPB14 VH-L-----V-----DED-----L-----V-----DEAV-----  
 DPB15 VY-----Q-Y-----DED-----L-----R-----V-----  
 DPB16 -----V-----DED-----E-----V-----DEAV-----  
 DPB17 VH-L-----V-----DED-----E-----DEAV-----  
 DPB18 VY-----V-----DED-----E-----V-----  
 DPB19 -----V-----E-----DE-----I-----DEAV-----  
 DPB20 VH-L-----V-----DED-----L-----FM-R-----EV-KV---K---ME-LIR---  
 SXB -SVY-E-----VVDGL-----YVH---A---L---M-----IG---F-----

To detect and distinguish between DP alleles in a practicable and economic fashion, however, one must know the nucleotide sequence of the alleles. Portions of the nucleotide sequences of various DPalpha and DPbeta alleles are shown below; the sequences are identified as above. The illustrative primers of the invention enable production of DNA from which the sequence of codons 8 to 90 can be determined. The location of allele sequences that are the preferred target sequences for hybridization with the various probes of the invention are designated as | -A- |, | -B- |, | -C- |, | -D- |, | -E- |, and | -F- |.

	+10	+15	+20	+25
	AsnTyrLeuPheGlnGlyArgGlnGluCysTyrAlaPheAsnGlyThrGlnArgPheLeuGluArgTyr			
	AGAATTACCTTTCCAGGACCGCAGGAATGCTACGCGTTTAATGGGACACAGCGCTTCCTGGAGAGATAC			
DPB4.1:	-----	-----	-----	-----
DPB4.2:	-----	-----	-----	-----
DPB2.1:	-----	-----	-----	-----
DPB2.2:	-----	-----	-----	-----
DPB8:	-----	-----	-----	-----
DPB5:	-----	-----	-----	-----
DPB7:	-----	-----	-----	-----
DPB3:	G-G-A---TT-	-----	-----	-----
DPB6:	G-G-A---TT-	-----	-----	-----
DPB11:	G-G-A---TT-	-----	-----	-----
DPB12:	-----G-GCA---TT-	-----GC-	-----A-	-----T-
DPB13:	G-G-A---TT-	-----	-----	-----
DPB1:	G-G-A---TT-	-----	-----	-----
DPB9:	G-GCA---TT-	-----	-----	-----
DPB10:	G-GCA---TT-	-----	-----	-----
DPB14:	G-GCA---TT-	-----	-----	-----
DPB15:	G-G-A---TT-	-----	-----	-----
DPB16:	-----	-----	-----	-----
DPB17:	G-GCA---TT-	-----	-----	-----
DPB18:	G-G-A---TT-	-----	-----	-----
DPB19:	-----	-----	-----	-----
DPB20:	G-GCA---TT-	-----	-----	-----
	-----A-----			



	+30	+35	+40	+45	+50
DPB4.1:	IleTyrAsnArgGluGluPheAlaArgPheAspSerAspValGlyGluPheArgAlaValThrGluLeu				
DPB4.2:	ATCTACAACCGGGAGGAGTTCCGCGCGCTTCGACACGCGACGTGGGGAGTTCGGGGCGGTGACGGAGCTG				
DPB2.1:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB2.2:	-----C-----	-----C-----	-----C-----	-----C-----	-----C-----
DPB8:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB5:	-----C-----	-----C-----	-----C-----	-----C-----	-----C-----
DPB7:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB3:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB6:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB11:	-----C-----	-----A-----	-----A-----	-----A-----	-----A-----
DPB12:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB13:	-----A-----	-----A-----	-----A-----	-----A-----	-----A-----
DPB1:	-----A-----	-----A-----	-----A-----	-----A-----	-----A-----
DPB9:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB10:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB14:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB15:	-----C-----	-----A-----	-----A-----	-----A-----	-----A-----
DPB16:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB17:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB18:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----
DPB19:	-----T-----	-----T-----	-----T-----	-----T-----	-----T-----

	+55	+60	+65	+70
	GlyArgProAlaAlaGluTyrTrpAsnSerGlnLysAspIleLeuGluGluLysArgAlaValPro			
	GGCGGCCTGCTGCGGAGTACTGGAACAGCCAGAGGACATCCTGGAGGAGAGCGGCCAGTGCCCG			
DPB4.1:	-----A--A-----			
DPB4.2:	-----A--A-----			
DPB2.1:	-----AG-----			-----G-----
DPB2.2:	-----A--A-----			-----G-----
DPB8:	-----AG-----			
DPB5:	-----			
DPB7:	-----			
DPB3:	-----A--A--C-----			
DPB6:	-----A--A--C-----			-----G-----
DPB11:	-----		-----C-----	-----G-----
DPB12:	-----A--A--C-----			-----G-----
DPB13:	-----			-----G-----
DPB1:	-----			
DPB9:	-----A--A--C-----			-----G-----
DPB10:	-----A--A-----			-----G-----
DPB14:	-----A--A--C-----		-----C-----	
DPB15:	-----		-----C-----	-----G-----
DPB16:	-----A--A-----			-----G-----
DPB17:	-----A--A--C-----			-----G-----
DPB18:	-----A--A-----			
DPB19:	-----AG-----			-----G-----
DPB20:	-----A--A--C-----		-----C-----	

|---C---| |---D---|

	+75	+80	+85	+90
AspArgMetCysArgHisAsnTyrGluLeuGlyGlyProMetThrLeuGlnArgArg				
GACAGGATGTCAGACACAACTACGAGCTGGCGGCCCATGACCCCTGCAGCGCCGAG				
DPB4.1:	-----	-----	-----	-----
DPB4.2:	-----	-----	-----	-----
DPB2.1:	-----	-----	-----	-----
PB2.2:	-----	-----	-----	-----
DPB8:	-----G-A-----	-----A-A-G-G-----	-----	-----
DPB5:	-----	-----A-A-G-G-----	-----	-----
DPB7:	-----G-A-----	-----A-A-G-G-----	-----	-----
DPB3:	-----G-A-----	-----A-A-G-G-----	-----	-----
DPB6:	-----	-----A-A-G-G-----	-----	-----
DPB11:	-----	-----A-A-G-G-----	-----	-----
DPB12:	-----G-A-----G-----	-----A-A-G-G-----	-----	-----
DPB13:	-----A-----	-----A-A-G-G-----	-----	-----
DPB1:	-----G-A-----	-----A-A-G-G-----	-----	-----
DPB9:	-----G-A-----	-----A-A-G-G-----	-----	-----
DPB10:	-----G-A-----	-----A-A-G-G-----	-----	-----
DPB14:	-----G-A-----	-----A-A-G-G-----	-----	-----
DPB15:	-----	-----T-----	-----	-----
DPB16:	-----	-----A-A-G-G-----	-----	-----
DPB17:	-----	-----A-A-G-G-----	-----	-----
DPB18:	-----	-----T-----	-----	-----
DPB19:	-----A-----	-----A-A-G-G-----	-----	-----
DPB20:	-----	-----A-A-G-G-----	-----	-----

I-E-I |-----F-----

The DNA sequences provided above are an important aspect of the present invention. Although only one strand of the sequence is shown, those of skill in the art recognize that the other strand of the sequence can be inferred from the information depicted above. This information enables the construction of the probes of the invention.

5 Many illustrative probes of the invention are shown in the Examples below. However, suitable SSO probes for hybridization analysis of the DPbeta alleles will comprise (or be complementary to) certain polymorphic sequences. Six sets of illustrative probes of the invention are depicted below; each set is designed to distinguish between the polymorphisms in a specific segment of the second exon of the HLA DPbeta gene. The

10 designation of the segments is as described above. The polymorphic residues encoded within the allelic variant in the segment to which a probe hybridizes is shown in one letter amino acid code (the dash means that prototypic residues are present in those positions) to the left of the probe sequence. The probes span the regions encoding the polymorphic amino acid residues and are shown as having a length of about 18 nucleotides. Those

15 sequences in the probe that encode the polymorphic amino acid residues, and thus must be included within a probe for detecting the alleles that encode the designated segment, are between the slash marks in the sequence. The DP alleles with which the probe will hybridize are shown to the right of the probe.

<u>HLA DPbeta SSO Probes</u>			
	<u>Amino Acid</u>	<u>Probe Sequence</u>	<u>DPbeta Alleles</u>
5	Segment A: LF-G:	TACCTTTTCCAGGGACGG	2.1, 2.2, 4.1, 4.2, 5, 7, 8, 19
	VY-L:	TACGTGTACCAAGTTACGG	3, 6, 11, 13
	VY-G:	TACGTGTACCAAGGGACGG	1, 15, 18
	VH-L:	TACGTGCACCAAGTTACGG	9, 10, 12, 14, 17, 20
10	Segment B: E-FA:	CGGAGAGGAGTTCGACGCGC	4.1, 7
	E-FV:	CGGAGAGGAGTTCGTGCGC	2.1, 3, 4.2, 6, 8, 9, 10, 12, 14, 16, 17, 18, 19, 20
	E-LV:	CGGAGAGGAGCTCGTGCGC	2.2, 5
	Q-YA:	CGGACAGGAGTACGACGCGC	11, 15
	E-YA:	CGGAGAGGAGTACGACGCGC	1, 13
	E-FV:	CGGAGAGGAATTCGTGCGC	10
15	Segment C: AAE:	CCTGCTGCGGAGTACTGG	1, 4.1, 7, 11, 13, 15
	DEE:	CCTGATGAGGAGTACTGG	2.1, 4.2, 8, 10, 16
	EAE:	CCTGAGGCGGAGTACTGG	2.2, 5, 19
	DED:	CCTGATGAGGACTACTGG	3, 6, 9, 12, 14, 17, 20
20	Segment D: I---K:	GACATCCTGGAGGAGAAAG	1, 4.1, 4.2, 5, 7, 18
	I---E:	GACATCCTGGAGGAGGAGAG	2.1, 2.2, 8, 9, 10, 12, 13, 16, 17
	L---K:	GACCTCCTGGAGGAGAAAG	3, 14, 20
	L---E:	GACCTCCTGGAGGAGGAGAG	6
	L---R:	GACCTCCTGGAGGAGAGAG	11, 15
	Segment E: M:	GACAGGATGTGCAGACAC	2.1, 2.2, 4.1, 4.2, 5, 6, 11, 15, 16, 17, 18, 20
25	V:	GACAGGAGTATGCAGACAC	1, 3, 7, 8, 9, 10, 12, 14
	Segment F: GGPM:	CTGGAGCGGGCCCAATGACC	2.1, 2.2, 4.1, 4.2
	DEAV:	CTGGACGAGGCCGATGACC	1, 3, 5, 6, 7, 8, 9, 10, 11
	VGPM:	CTGGTCCGGGCCCAATGACC	15, 18

Because the probes of the invention are single stranded for use in hybridization, it is important to note that merely because a probe is designed to hybridize with, for example, the coding strand, does not mean that an equally useful probe could not be designed that would hybridize to the complementary sequence present on the noncoding strand.

The sequence information provided above also relates to other important aspects of the invention. The preferred primers of the invention are designed to amplify many different DP alleles. In many instances, as demonstrated in the Examples below, such primers are very useful. However, those of skill in the art recognize that the DNA

sequence information provided above can also be used to design primers that will enable allele specific amplification. Such allele-specific primers will only amplify a single allele or a certain subset of known alleles. For example, the "DEAV" probes of the invention can be used as one primer of a primer pair to provide for allele-specific amplification of "DEAV" DPbeta alleles.

The present invention also relates to kits that comprise a multicontainer unit comprising essential components for practicing the present method. For example, the kit can contain primers for PCR, as such primers are necessary in the preferred embodiment of the invention. These primers will amplify at least the DPbeta gene, and, when appropriate, for example in forensic analysis, primers can be included that also amplify the DPalpha gene. The kit must also contain SSO probes for at least the DPbeta gene, and, when appropriate, for the DPalpha gene as well. In some cases, the SSO probes may be fixed to an appropriate support membrane which is useful for the hybridization analysis. Other optional components that may be contained in containers within the kit include, for example, an agent to catalyze the synthesis of primer extension products, the substrate nucleotides, means used to label (for example, an avidin-enzyme conjugate and enzyme substrate and chromogen if the label is biotin), and the appropriate buffers for PCR or hybridization reactions. In addition to the above components, the kit can also contain instructions for carrying out the present method.

A number of examples of the present invention, which are provided only for illustrative purposes and not to limit the scope of the invention, are presented below. Numerous embodiments of the invention within the scope of the claims that follow the examples will be apparent to those of ordinary skill in the art from reading the foregoing text and following examples. In the following examples, certain techniques were standard, unless specifically indicated otherwise. Such techniques include PLT, which was performed essentially as described by Shaw *et al.*, 1980, *J. Exp. Med.* 152:565-580. Generally, for lymphocyte priming, responder and stimulator cells were thawed, washed, and resuspended in RPMI-1640 medium supplemented with glutamine and antibiotics (complete media). Responding cells were mixed with irradiated stimulator cells in a ratio of 2:1, and the cell mixture was incubated for ten days at 37 degrees C. The primed irradiated stimulator cells were co-cultured with irradiated stimulator cells in complete media. After 48 hours, <sup>3</sup>H-thymidine was added to the culture. The cells were harvested 18 hours later, and tritium incorporation was evaluated by counting beta-emission.

DNA sequence analysis was performed as described by Maniatis et al., Molecular Cloning: A Laboratory Manual (New York, Cold Spring Harbor Laboratory, 1982). Generally, the sequences to be analyzed were cloned into an M13 cloning vector and analyzed by either the Maxam-Gilbert technique or by the dideoxy chain termination technique. Synthetic oligonucleotides, both primers and probes, were synthesized using commercially available instruments and techniques well known in the art.

### Example 1

#### Analysis of the DNA Sequences of HLA DP Alleles

The DNA sequences of the variable second exons of a variety of alleles of the DPalpha gene and of the DPbeta gene were determined. The DNA samples used were chosen to represent as wide a spectrum of PLT defined DP alleles as possible. DNA was extracted from cell lines homozygous for the standard six DPw types, from cells exhibiting unusual typing reactions, and from cells exhibiting DP blank reactions. DNA extraction was by standard techniques, as described by Maniatis et al., in Molecular Cloning: A Laboratory Manual. The variable second exons of the DPalpha and the DPbeta genes were amplified by the PCR method, as described. The amplified DNA sequences were cloned into an M13 derived vector, and the DNA sequences were determined by the chain termination method. The cell lines used, their DR serotypes, their PLT defined DPw types, and the DNA defined alleles they were found to contain are listed in tabular form below (blank spaces indicate data not determined).

	<u>Cell Line</u>	<u>DR Type</u>	<u>DPw Type</u>	<u>DPB Alleles</u>	<u>DPA Alleles</u>
5	CRK	7	1*	1, 11	
	RSO101	w6	1, 3	1, 3	
	BMG	4	1, 4	4.1	
	QBL	3	2	2.2	1
	WJR	2	2	2.1	
10	PJM	3	2, 3	2.1, 3	
	RMD	5, w13	2, 4	2.1, 4.1	
	JMOS	4, 5	2, 6	2.1, 6	
	SLE	w13	3	3	1
	COX	3	3	3	1
15	OPR	w8, w10	3, 4	3, 4.1	
	JAH	4	3, 4	3, 4.1	
	MRI	1, 2	3, 5	3, 5	
	HHK	w6	4	4.1	
	LB1	3, 7	4, MAS	1, 4, 4.2	
20	APD	w6	4*	2, 4	
	LUY	w8	1, 4	1, 4.1	1, 2
	WDV	w6	2, 4	2.1, 4.1	
	SMF	2, w12	4, 5	4.1, 5	
	BCR	4, w6	4, 6	4.1, 6	
25	GTER	w8, 9	5	5	
	HAS	4	5	5, 19	
	DKY	9	5	2.1	1
	BIN40	4	3, 6	3, 6	1
	LG2	1	?	4.1	
30	PIAZ	2.7		8	
	TOK	2		9	2
	BM21	w11		10	
	VLA	2, 3		11	
	GBA	1, 7		11	
35	CD1	3, 4	3, 6	10	
	CD2	7, (5)	2, 4	4.2, 10	
	CD8	3, 7	4	4.1, 4.2	
	CD11		2	2.1, 4.2	

DP "MAS" is provisional designation for a newly defined DP specificity related to the DPB4.2 allele. "CD" cell lines actually are samples from CD patients.

\* - refers to cells with unusual DPw phenotypes.



As shown from the foregoing, the DNA analysis of the HLA DP subtypes shows that specific sequences correlate with the known PLT-defined DPw typings, indicating that the polymorphic epitopes recognized by the primed T cells are on the DPbeta chain. For some DP types, e.g., DPw2 and DPw4, sequence analysis has revealed subtype variants. The variants for DPw2 have been designated DPB2.1 and DPB2.2; cells typed for PLT as DPw4 "new" (e.g., LB1) or DPw4\* (e.g., APD) contain the more rare DPB4.2 subtype. The DPB4.2 subtype is more related by sequence to the DPB2.1 allele than to the DPB4.1 allele. Individual CD11 is PLT typed as DPw2, but contains the closely related DPB2.1 and DPB4.2 alleles.

The results above also show that unique DPbeta sequences correspond to the DPw1, DPw3, DPw5, and DPw6 specificities, and these alleles have been designated to reflect this correlation. A few exceptions are, however, that cell line DKY has been typed as DPw5, but contains the DPB2.1 allele, and that individual CD2 is PLT typed as DPw2, but contains the DPB4.2 and DPB10 alleles.

### Example 2

#### PCR Amplification of the DPalpha and DPbeta Genes

The DPalpha and DPbeta genes of some of the cells described in Example 1 were amplified by PCR. The primers used, which were synthetic, are shown below. In this depiction, the left side primers, GH98 and DB01, are from the upper strand, and direct DNA polymerase to extend rightward. The right side primers, GH99 and DB03, are from the lower strand, and direct synthesis leftward. The areas of the genes to which the primers bind, and which will act as templates for primer extension are also shown. Lower case letters indicate bases in the primer that are not complementary to the target genomic DNA (shown in the opposite strand). These changes in the primers incorporate restriction enzyme sites (BamHI or PstI) at the ends of the amplified DNA and facilitate cloning of the amplified DNA. The oligonucleotide primers GH98 and GH99, which are used for the amplification of the second exon of DPalpha, amplify a 243 bp segment. The first two bp of the PCR product are from the intervening sequence which flanks the exon. The oligonucleotide primers DB01 and DB03 amplify a 294 bp segment of the second exon of DPbeta. The left 13 bp and the right 17 bp of the product are from the intervening sequence.

DPalpha Primers

----- GH98 -----> Phe Val Gln Thr His Arg Pro Thr ... Leu Asn Asn Asn Leu Asn Thr Leu Ile  
 15  
 CGCGATCCTGTGTCAACTTATGCCGG TTT GTA CAG ACG CAT AGA CCA ACA ... TTG AAC AAC AAC TTG AAT ACC TTG ATC CAGCGTTCCAACACACACTCAGGCCAC  
 TCGCCTGGTACACAGTTGAATACGGCG AAA CAT GTC TGC GTA TCT GGT TGT ... AAC TTG TTG AAC TTA TGG AAC TAG GTCGCAAGGTTGGTGTGAcgtCGGtc  
 70  
 <----- GH99 ----->

DPbeta Primers

----- DB01 -----> Leu Phe Gln Gly Arg Gln Glu Cys Tyr ..... Glu Leu Gly Gly Pro Met Thr Leu Gln  
 10 15 85 90  
 CagggatCCGACAGAGATTAC CTT TTC CAG GGA CGG CAG GAA TGC TAC ..... GAG CTG GGC GGC CCC ATG ACC CTG CAG CGCCGAGGTGAGTGAGGGCTTTGG  
 GGGAGGGGCGCTCTCTTAATG GAA AAG GTC CCT GCC GTC CTT ACG ATG ..... CTC GAC CCG CCC GGG TAC TGG GAC GTC GCGGCTCCACTCACTgaCGtcctg  
 <----- DB03 ----->

Hybridization of the primers and the synthesis of the elongated primer containing products were essentially as described in EP 258,017 and in the protocols provided by the manufacturer, PECL, of the Thermal Cycler used to perform the PCR. Amplification was for 28 cycles; however, more, i.e., 35, cycles can on occasion yield better results.

Two other illustrative primers of the invention for amplifying the second exon of DPbeta alleles are designated UG19 and UG21. The sequences of these primers and the other illustrative DPbeta primers of the invention are shown below.

<u>Designation</u>	<u>DPbeta Primers Sequence</u>	<u>Side</u>
DB01	CAGGGATCCGCAGAGAATTAC	Left
DB03	GTCCTGCAGTCACTCACCTCGGCG	Right
UG19	GCTGCAGGAGAGTGGCGCCTCCGCTCAT	Left
UG21	CGGATCCGGCCCAAGCCCTCACTC	Right

### Example 3

#### SSO Probes for Hybridization Analysis of DPbeta Alleles

Illustrative probes of the invention referred to throughout the remainder of the Examples are described below in tabular form. In the Table, the probe designation, probe sequence, polymorphic amino acid sequence encoded in the region of the allele to which the probe hybridizes, segment designation, and hybridization and wash conditions are shown. Probes are shown as having a  $^{32}\text{P}$  or "X" label, where X represents HRP, as discussed in the Examples below. Where a probe sequence is indicated by an X followed by a probe designation, the sequence of the probe is identical to that of the probe designated after the X, except that the  $^{32}\text{P}$  label has been replaced by an HRP label.

Those of skill in the art recognize that depending on the type of label used, hybridization and wash conditions will differ. Although, in a preferred embodiment, the probes will be labeled nonisotopically (e.g., with HRP), some of the probes have been used with isotopic (e.g.,  $^{32}\text{P}$ ) labels. Consequently, hybridization and wash conditions for  $^{32}\text{P}$ -labeled and HRP-labeled probes are shown, where such conditions have been empirically determined (see Bugawan *et al.*, 1988, *J. Immunol.* 141(12):4024-4030). In the Table, the conditions referred to assume a hybridization solution composed of 5 x Denhardt's solution, 0.5% SDS, and the indicated amounts (i.e., 0.1 x, 3 x, 5 x) of SSPE. Five x Denhardt's solution contains 0.5 g Ficoll, 0.5 g polyvinylpyrrolidone, 0.5 g BSA (Pentax Fraction V) per 500 ml. The wash solution contains 0.1 x SSPE and 0.1% SDS

(for HRP-labeled probes, 0.1% Triton X-100 was used in place of SDS); the wash step is carried out at the indicated temperature for ten minutes. As described in Example 11, however, the use of tetramethyl ammonium chloride, or similar salts, can be used to allow for more uniform hybridization and wash conditions, a preferred condition when a number  
5 of probes are used in a panel to determine the types of DP alleles in a sample.

SSOs for HLA DPbeta Typing

	<u>Probe Designation</u>	<u>Amino Acid Sequence</u>	<u>Probe Sequence</u>	<u>Region</u>	<u>Hybridiz'n/Wash Conditions</u>
5	DB10	LFQG	32P-GAATTACCTTTTCCAGGGA	A	5 x @ 50°/42° H <sub>2</sub> O bath
	DB27	LFQG	X-DB10	A	5 x @ 50°/42° air
	DB11	VYQL	32P-ATTACGTGTACCAGTTACG	A	3 x @ 55°/42° H <sub>2</sub> O bath
	DB28	VYQL	X-DB11	A	3 x @ 55°/42° H <sub>2</sub> O bath
	DB58	VYQL	X-ATTACGTGTACCAGTTA	A	3-5 x @ 42°/42° air
10	DB23	VYQL	X-CGTAACCTGGTACACGTAAT	A	5 x @ 50°/42° H <sub>2</sub> O bath
	DB36	VYQL	X-DB23	A	5 x @ 50°/42° air
	DB12	VYQG	32P-CGTCCCTGGTACACGTAAT	A	5 x @ 50°/42° H <sub>2</sub> O bath
	DB29	VYQG	X-DB12	A	5 x @ 50°/42° H <sub>2</sub> O bath
	DB22	VHQL	32P-ATTACGTGCACCAGTTACG	A	3 x @ 55°/42° H <sub>2</sub> O bath
20	DB35	VHQL	X-DB22	A	3 x @ 50°/42° H <sub>2</sub> O bath
	DB13	AAE	32P-CCTGCTGCGGAGTACTG	C	3 x @ 55°/42° H <sub>2</sub> O bath
	DB30	AAE	X-DB13	C	3 x @ 50°/42° H <sub>2</sub> O bath
	DB14	DEE	32P-CAGTACTCCTCATCAGG	C	5 x @ 42°/42° H <sub>2</sub> O bath
	DB31	DEE	X-DB14	C	5 x @ 42°/42° H <sub>2</sub> O bath
25	DB16	EAE	32P-CAGTACTCCGCCTCAGG	C	5 x @ 42°/42° H <sub>2</sub> O bath
	DB32	EAE	X-DB16	C	3-5 x @ 42°/42° H <sub>2</sub> O bath
	DB59	EAE	X-CCTGAGGCGGAGTACTG	C	5 x @ 50°/42° H <sub>2</sub> O bath
	DB17	DED	32P-CCTGATGAGGACTACTG	C	5 x @ 50°/42° H <sub>2</sub> O bath
	DB33	DED	X-DB17	C	5 x @ 50°/42° air
30	DB18	I-K	32P-GACATCCTGGAGGAGAAGCD	D	0.1x @ 55°/42° H <sub>2</sub> O bath
	DB34	I-K	X-DB18	D	3 x @ 55°/42° H <sub>2</sub> O bath
	DB19	I-E	32P-GCTCCTCCTCCAGGATGTC	D	5 x @ 50°/42° H <sub>2</sub> O bath
	DB37	I-E	X-DB19	D	5 x @ 55°/42° H <sub>2</sub> O bath
	DB20	L-K	32P-GACCTCCTGGAGGAGAAGCD	D	3 x @ 55°/42° H <sub>2</sub> O bath
35	DB38	L-K	X-DB20	D	5 x @ 55°/42° H <sub>2</sub> O bath
	DB21	L-E	32P-GCTCCTCCTCCAGGAGGTC	D	5 x @ 50°/42° H <sub>2</sub> O bath
	DB39	L-E	X-DB21	D	5 x @ 50°/42° air
	DB62	L-E	X-GACCTCCTGGAGGAGGAG	D	3 x @ 50°/42° H <sub>2</sub> O bath
	DB63	L-R	X-GACCTCCTGGAGGAGAGG	D	3 x @ 50°/42° H <sub>2</sub> O bath
40	DB25	GGPM	32P-CTGCAGGGTCATGGGCC- CCCG	F	5 x @ 50°/42° H <sub>2</sub> O bath
	DB40	GGPM	X-DB25	F	3 x @ 50°/42° H <sub>2</sub> O bath
	DB26	DEAV	32P-CTGCAGGGTCACGGCCT- CGTC	F	5 x @ 50°/42° H <sub>2</sub> O bath
	DB41	DEAV	X-DB26	F	3 x @ 50°/42° H <sub>2</sub> O bath

With reference to the Table above, one should note that because DB28 cross-hybridizes with DB32, superior results can be obtained using probes DB58 and DB59 in place of DB28 and DB32, respectively. In addition, probe DB63 is preferred over DB39.

Example 4SSO Probes for Hybridization Analysis of DPalpha Alleles

Examples of suitable SSO probes for hybridization analysis of DPalpha alleles are shown below. Two sets of probes are illustrated; each set is designed to distinguish between the polymorphisms in a specific segment of the second exon of HLA DPalpha gene. ASO1 and ASO2 bind to the DPA1 allele at the region containing the polymorphic segments containing methionine (M) and glutamine (Q), respectively. ASO3 and ASO4 bind to the DPA2 allele to the region containing the polymorphic segments which contain glutamine and arginine (R), respectively. ASO2 and ASO4 distinguish the polymorphic segment containing glutamine from that containing arginine. The probes span the regions encoding these polymorphic amino acid residues. Hybridization using these probes is usually carried out in a solution containing 5 x SSPE, 5 x Denhardt's, and 0.5% SDS, for at least 1 hour at 42°C. Also shown are the washing conditions for use with the probes.

HLA DPalpha SSO Probes

<u>Probe</u>	<u>Sequence</u>	<u>Washing Conditions</u>
ASO1	AGATGAGATGTTCTATG	2 x SSPE, 0.1% SDS, 42°
ASO2	GTTTGGCCAAGCCTTTT	2 x SSPE, 0.1% SDS, 50°
ASO3	AGATGAGCAGTTCTATG	2 x SSPE, 0.1% SDS, 50°
ASO4	GTTTGGCCGAGCCTTTT	2 x SSPE, 0.1% SDS, 55°

Example 5Analysis of Amplified DPbeta Sequences by Hybridization with SSO Probes

PCR amplified DPbeta sequences from 24 HTC's were analyzed in a dot blot format with a panel (n=9) of <sup>32</sup>P-labeled SSO probes, and the DP type was inferred from the pattern of probe binding. The extraction of DNA from the cells was as described in Example 1. The target regions of the cellular genome, i.e., the second exon of the DPbeta genes, were amplified by the PCR technique as described in Example 2, except that the DNA was from the cells listed in tabular form above.

The amplified DNAs were dot blotted onto a filter; a separate filter containing the panel of samples was prepared for analysis by hybridization with each SSO probe. To dot blot the samples, five microliters of each amplified sample were diluted with 195 microliters of a solution containing 0.4 N NaOH and 25 mM EDTA and spotted onto 9

replicate Genatran 45 (Plasco) nylon filters by first wetting the filters with water, placing them in a Bio-dot (Bio-Rad, Richmond, CA) apparatus for preparing dot blots, applying the samples, and rinsing each well with 0.4 ml of 20 x SSPE (3.6 M NaCl, 200 mM  $\text{NaH}_2\text{PO}_4$ , and 20 mM EDTA). The filters were removed, rinsed in 2 x SSPE, and baked for 30 minutes at 80°C in a vacuum oven.

The samples on the filters were hybridized with SSO probes of the invention. Hybridization was with 0.25 to 0.5 pmoles of probe in 2 to 5 ml of hybridization solution. Hybridization and wash conditions were as described in tabular form in Example 3. The results of this DPbeta typing are shown below, which also shows the probe with which the samples on the filter were hybridized and the encoded amino acid sequence detected by the probe.

## DPbeta Oligonucleotide Probes

CELL LINE	HLA DR	HLA DPw	A			C			D			DPbeta Allele type
			DPI0 LFQG 4,2,5, 7,8	DBI1 VYQL 6,3,11	DBI2 VYQG 1	DBI3 AAE 4,1,7, 1,11	DBI4 DEE 4,2,8, 2,1,10	DBI7 DED 6,3,9, 12	DBI8 I-K 4,5,7, 1	DBI9 I-E 2,8,9, 10,12	DB20 L-K 3	
*1 COX	3	3	-	+	-	-	-	+	-	-	+	3
2 950	3	1	-	-	+	+	-	-	+	-	-	1
*3 BM21	11	blank	-	+/-c	-	-	+	-	-	+	-	10
4 BM16	12	2	+	-	-	-	+	-	-	+	-	2.1 (or 8)
5 BR1G	2	2	+	-	-	-	+	-	+	+	-	2.1/4.2 or 4.2/8
6 JVM	5	2	+	-	-	-	+	-	-	+	-	2.1 (or 8)
7 WT46	6	2	+	-	-	-	+	-	-	+	-	2.1 (or 8)
8 MANN	7	2	+	-	-	-	+	-	-	+	-	2.1 (or 8)
9 J640	4	3	+	-	-	-	+	-	-	+	-	3
10 SLE	13	3	-	+	-	-	-	+	-	-	+	3
11 JMF	7	4	-	+	-	-	-	+	-	-	+	3
12 BGE	2	4	+	-	-	+	-	-	+	-	-	4.1 (or 7)
13 BM14	4	4	+	-	-	+	-	-	+	-	-	4.1 (or 7)
14 BM92	4	4	+	-	-	-	+	-	+	-	-	4.2
15 TS10	4	4	+	-	-	+	-	-	+	-	-	4.1 (or 7)
16 RLO	4	4	+	-	-	+	-	-	+	-	-	4.1 (or 7)
*17 APD	6	4	+	-	-	-	+	-	+	-	-	4.2
18 BUP	7	4	+	-	-	+	+	-	+	-	-	4.1/4.2
19 MMR	4	4	+	-	-	+	-	-	+	-	-	4.1 (or 7)
*20 LUY	8	4/1	+	-	+	+	-	-	+	-	-	1/4.1 (or 7)
21 YOS	4	4	+	-	-	-	+	-	+	-	-	4.2
*22 DKY	9	5	+	-	-	-	+	-	-	+	-	2.1 (or 8)
23 HAS	4	5	+	-	-	-	-	-	+	-	-	5
*24 TOK	2	blank	-	-	-	-	-	+	-	+	-	9

\* Cell line DPbeta type confirmed by DNA sequencing. +/- designates weak signal. Superscript c indicates the presence of an additional polymorphic sequence in region A encoding the residues VHYL, which cross-hybridizes with the DBI1 probe (no cross-hybridization occurs with probe DB22). Superscript d represents cross-hybridizing due to the ability of the DBI1 probe to bind to sequences recognized by the DBI0 probe (cell line BM92) or due to the ability of the DBI9 probe to bind to sequences recognized by the DBI8 probe (cell lines YOS and HAS).



The determination of DPbeta types of the cells based upon the hybridization analysis with the SSO probes has been discussed above. To determine the DPbeta type of a sample, the binding of the probes to the sample was examined. The alleles present were inferred from the pattern of probe binding. For example, sample 1 formed hybrids with SSO probes DB11, DB17, and DB20. The amino acids encoded by DB11, DB17, and DB20 are VYQL, DED, and LEEK, respectively. An examination of segments A, C, and D of the DPbeta allelic amino acid sequences shows that the sequence VYQL is present in DPB6, DPB11, and DPB13; the sequence DED is present in DPB17, DPB14, DPB12, DPB9, DPB6, and DPB3; the sequence LEEK (L\_\_\_\_K) is present in DPB18, DPB14, DPB4.1, DPB4.2, DPB5, DPB7, DPB3, and DPB1. The only allele which contains the three sequences which hybridize with the probe is DPB3. Thus, the DP type of sample 1 based on SSO typing is DPB3.

The DPB types of the other samples were inferred by the same type of analysis, and the determined types are depicted above. In the depiction, the asterisk (\*) indicates cells where the DPbeta genotype was also determined by sequence analysis. The DPw type of the cell lines COX (formerly w1→w3) and BM21 (formerly w1→blank) have recently been changed to the type indicated. The symbol ± refers to a weak signal obtained with a probe. In some cases (BM21 and TOK), this weak signal reflects the presence of an additional polymorphic sequence in region A encoding the amino acid residues VHYL, which cross-hybridizes to the DB11 probe. The sequence can be more conveniently typed with region A probe DB22. For other cell lines (BM92), there is apparently a background cross-hybridization of the DB11 probe to the sequences recognized by the DB10 probe. In similar fashion, cross-hybridization signals can occur with the DB19 probe on sequences complementary to the DB18 probe.

The panel of SSO used in this Example detects variation at only 3 of the 5 polymorphic regions and does not detect all allelic variants at these 3 regions. The typing system using the procedure of this example is simple and unequivocal for HTCs, but given the patchwork pattern of polymorphism, can occasionally give rise to ambiguous typing for heterozygous individuals if the hybridization pattern of the various probes can be interpreted as more than a unique pair of alleles. This ambiguity arises from the many different combinations of the DPbeta sequence variants that constitute the different DPbeta alleles. However, with the use of the additional SSO probes provided by the invention,

which additional probes span the remaining polymorphic regions, unambiguous typings can be obtained for heterozygous individuals.

#### Example 6

##### HLA DP Typing of Coeliac Disease Patients

##### by DNA Sequence Analysis of PCR Amplified Target Regions

The cells of four patients with coeliac disease (CD) were PLT-typed, and the DNA sequences of the second exon of the DPbeta determined as described in Example 1. The CD diagnosis was based on clinical symptomology.

The results of the analysis obtained by DNA sequencing of the DPbeta (second exon) of the CD cells has been discussed above. From these results, one observes that, when CD cells are compared to non-CD cells, there is an apparent increase in the frequency of the DPB4.2 allele. In addition, the DPB10 allele sequence is present in two independent CD patients yet observed in only one of the 30 non-CD cell lines.

#### Example 7

##### HLA DP Typing of Coeliac Disease Patients

##### by SSO Probe Hybridization Analysis

The cells of 19 patients with CD and of 43 non-CD controls were analyzed for HLA DP type by SSO probe hybridization analysis. The diagnosis of CD was based on clinical symptomology; the CD patients as well as control individuals were all from Italy. DNA extraction was as described in Example 1. PCR amplification of the samples was as described in Example 2. Analysis of the amplified sequences was as described in Example 4.

The results of the analysis showed that there was a significant increase in the DPB4.2 allele in CD patients as compared to non-CD controls; this allele was present in 12 of 19 CD patients, but only 3 of 43 control patients. The DPB4.2 and DPB3 alleles were present in 17 of 19 CD patients and in 15 of 43 controls. The genotype DPB4.1/4.2 was present in 10 of 19 CD patients and in only 1 of 43 controls.

#### Example 8

##### HLA DP Typing of Forensic Samples

Samples which contain genomic nucleic acids of the suspect individual are obtained. The target regions of the genome, i.e., the regions containing the second exon of

the DPalpha and DPbeta genes, are amplified by the PCR technique, as described in Example 2, except that the nucleic acids from the suspect replace those of the cells in the Example, and except that the amplified samples contain a  $^{32}\text{P}$ -label. The amplified sample is hybridized with the probes of the invention, which have been dot blotted onto the same filter, and which have been immobilized on the filter by poly-dT tails. The hybridization and washing conditions for the filter allow only perfectly matched hybrids to remain in duplex state. The filters are examined to determine which probes form hybrids with the labeled sample.

A sample which is to be compared with that obtained from the suspect is examined for DP type by the same procedure, i.e., by PCR amplification and hybridization with immobilized SSO probes. The pattern of binding to the SSO probes of the sample from the suspect and of the comparison sample is examined to determine the similarity or difference in the hybridization patterns.

#### Example 9

##### HLA DP Typing Using SSO Probes

##### Labeled with Horseradish Peroxidase

A panel of cells from 39 individuals was typed for DPbeta alleles using SSO probes for the second exon variants. The probes were labeled with horseradish peroxidase (HRP), and hybrids detected in a dot blot format.

The cells analyzed were from fourteen IDDM patients, five DR3 control non-IDDM patients, and nineteen HTC's which typed by PLT as DP blank. IDDM patients were identified by clinical symptomology. DNA was isolated from the cells as described in Example 1. The target region, i.e., the second exon of the DPbeta alleles, was amplified using the PCR technique in 200 microliters of reaction mixture that contained 50 mM Tris-HCl, pH 8.3; 2.5 mM  $\text{MgCl}_2$ ; 100 micrograms/ml gelatin; 0.75 mM in each of the four deoxynucleoside triphosphates; the primers DB01 and DB03; and Taq polymerase. The ramping for amplification was the following: heating for 30 seconds to  $94^\circ\text{C}$  followed by incubation at that temperature for 30 seconds; cooling for 1 minute to  $55^\circ\text{C}$  followed by incubation at that temperature for 30 seconds; heating to  $72^\circ\text{C}$  for 30 seconds followed by incubation at that temperature for 45 seconds. This cycling was repeated for 42 cycles. After amplification, the reaction mixtures were sampled and monitored by gel electrophoresis on gels containing 3% Nusieve, 1% Agarose to determine if all the DNA amounts were comparable.

Filters containing the dot blotted samples were prepared by blotting 150 microliters/dot of denatured amplified DNA on Genatran nylon membranes and UV treating the filters containing the samples for 5 minutes. The latter treatment is to fix the sample to the membranes. The amplified DNAs were denatured by treating 5 microliters of the PCR reaction mixture in a total volume of 150 microliters containing 0.4 N NaOH and 25 mM EDTA. Eight replicate filters were prepared for hybridization with HRP labeled ASO probes.

Prior to hybridization, the sample containing filters were incubated for 15 minutes in pre-hybridization solution (1 x SSPE, 5 x Denhardt's solution, 1% Triton X-100) without probe. In the pre-hybridization solution, Triton X-100 was used in place of SDS. Hybridization was in the same solution, which additionally contained 1 picomole probe/ml. Each filter was incubated for 40 minutes with 2.5 ml of hybridization solution containing one of the HRP labeled probes. The probes used were DB27, DB28, DB29, DB30, DB31, DB32, DB33, and DB35. The probes and hybridization conditions are listed in tabular form in Example 3. After hybridization, the filters were washed as stated in Example 3, under suitably stringent conditions, i.e., in 0.1 x SSPE, 0.1% Triton X-100 for 5 minutes at 42°C. The HRP-labeled SSO probes were prepared essentially by the methods disclosed in PCT publications 89/02931 and 89/02932, incorporated herein by reference.

These methods essentially involve derivatizing the nucleic acid probe using a linear linking molecule comprising a hydrophilic polymer chain (e.g., polyoxyethylene) having a phosphoramidite moiety at one end and a protected sulfhydryl moiety at the other end. The phosphoramidite moiety couples to the nucleic acid probe by reactions well known in the art (e.g., Beaucage *et al.*, 1988, *Tetrahedron Lett.* 22:1859-1862), while the deprotected sulfhydryl group can form disulfide or other covalent bonds with the protein, e.g., HRP. The HRP is conjugated to the linking molecule through an N-maleimido-6-aminocaproyl group. The label is prepared by esterifying N-maleimido-6-aminocaproic acid with sodium 4-hydroxy-3-nitrobenzene sulfonate in the presence of one equivalent of dicyclohexylcarbodiimide in dimethylformamide. After purification, the product is added to phosphate buffer containing HRP at a weight ratio of 8:1 HRP to ester. The oligonucleotide probe is synthesized in a DNA synthesizer, and the linking molecule having the structure  $(C_6H_5)_3CS-(CH_2CH_2O)_4-P(CH_2CH_2CN)[N(i-PR)_2]$  attached using phosphoramidite synthesis conditions. The trityl group is removed, and the HRP

derivative and probe derivative are mixed together and allowed to react to form the labeled probe. A biotin-labeled probe or primer may be prepared by similar methods.

Samples which contained hybridized probe were detected using a color development reaction, as described in Sheldon *et al.*, 1986 *Proc. Natl. Acad. Sci. USA* 83:9085-9089, which utilizes TMB/H<sub>2</sub>O<sub>2</sub>. The detection system is described in Example 10 below. The HLA DP genotypes of the amplified DNA samples were readily apparent from the filters.

#### Example 10

##### HLA DPbeta Typing with HRP Labeled SSO Probes

#### 10 A. PCR Amplification

DPB typing can utilize as many as or more than 14 SSO probes (sequence specific oligonucleotides), so amplification is carried out on 0.5 to two micrograms of DNA, in 200 microliters of reaction volume, if DNA is not limiting. Lower amounts of DNA, i.e., 100 ng, can be amplified, but more cycles of amplification should be performed with such samples, i.e., 45 cycles.

The PCR reaction is started by mixing the following by vortexing for 1 to 2 seconds.

	DNA	0.5 to 2 µg
	10 x Taq buffer	20 µl
20	100 mM dNTPs	1.5 µl
	DPB primer [10 µM UG19 or DB01]	10 µl
	DPB primer [10 µM UG21 or DB03]	10 µl
	Taq polymerase 5 U/µl	1.2 µl

Glass-distilled H<sub>2</sub>O is added to achieve a final volume of 200 µl. 10 X Taq salts are 500 mM KCl; 100 mM Tris, pH 8.3; 15 mM MgCl<sub>2</sub>; and 1 mg/ml gelatin. Negative controls (i.e., no DNA) should be included in each PCR run. Typically, 30-35 cycles of amplification in a Perkin-Elmer/Cetus Instruments DNA Thermal Cycler are sufficient. The cycles are designed to denature at 96°C for 30 seconds, and anneal and extend at 65°C for 30 seconds. If primer pair DB01/DB03 is used, annealing is at 55°C for 30 seconds and extending is at 72°C for 30 seconds. Analytical gels can be used to check PCRs and to quantitate amount of DNA to be used for dot-blots.

### B. Dot blot

Typically, 5  $\mu$ l of the amplified DNA contain approximately 200 ng, more than enough for a single dot blot. Remember, however, that as many as or more than 14 dots may be required, i.e., about 70  $\mu$ l of the amplification reaction would then be used in preparing the dot blots. For each 5  $\mu$ l of amplification reaction, 50  $\mu$ l of 0.4 N NaOH and 25 mM EDTA are added to the DNA. Five minutes is sufficient to complete denaturation of DNA. The Genatran membrane is first wetted in 2 x SSPE, and then the 150  $\mu$ l of denatured DNA are loaded into the dot blot apparatus. The membrane is rinsed in 2 x SSPE, and the DNA is fixed to the membrane with exposure to UV light for five minutes, i.e., by a 55 mJ/cm<sup>2</sup> exposure in a Stratalinker 1800™ UV light box, marketed by Stratagene.

### C. Hybridization

The membrane is again wetted in 2 x SSPE, and about 5 ml of hybridization solution per 8 x 12 cm membrane (size of dot blot apparatus) are added. About 1 to 1.5 picomoles of HRP probe are added per ml hybridization solution, and the probes are allowed to hybridize for at least one hour. Hybridization solution is SSPE (as indicated above), 5 x Denhardt's, and 1 % Triton X-100. The membranes are then washed with 0.1 x SSPE and 0.2% Triton X-100 for ten minutes. Otherwise, hybridization and wash conditions were as described in Example 3. The probes used were DB27, DB29, DB30, DB31, DB33, DB34, DB35, DB37, DB38, DB40, DB41, DB58, DB59, DB62, and DB63.

### D. Detection

The following steps for detection of probes are done at room temperature with moderate shaking and just enough solution to completely cover the membrane, as is described in Bugawan *et al.*, 1988, Bio/Technology 6:943-947. The detection is carried out by a 5 minute incubation of the membrane with Buffer B, a 5 minute was with Buffer C, and 10 minutes incubation under light exclusion with Buffer C and TMB (48 ml of Buffer C and 2.5 ml of 2 mg/ml TMB). Buffer B is 100 mM NaCl, 1 M urea, 5% Triton X-100, and 1 % dextran sulfate. Buffer C is 100 mM sodium citrate, pH 5.0. TMB is 3, 3', 5, 5'-tetramethylbenzidine. About 23  $\mu$ l of 3% H<sub>2</sub>O<sub>2</sub> are added to 50.5 ml of Buffer C/TMB; the resulting solution is used to develop the color on the membranes (color comes

up within 1-5 minutes) under light-excluding conditions. The color development is stopped by washing in H<sub>2</sub>O with a small amount of Buffer C. The wash is repeated twice for 30 minutes. Pictures of the membrane are taken and the membrane is stored in Buffer C under no light.

5        The methods described herein, as well as the SSO probes and primers, and kits containing them, are useful for the accurate, relatively simple, and economic determination of an individual's HLA DP genotype. Accurate DP typing will prove important in several medical applications. For example, accurate HLA DP matching of donors and recipients will be helpful in the prevention of allograft rejection and in the prevention of host versus  
10       graft disease. Because certain HLA DP genotypes appear to be linked to certain autoimmune diseases, including, for example, coeliac disease, myasthenia gravis, and IDDM, HLA DP DNA typing is useful in an early diagnosis of the disease, prior to manifestation of full clinical symptoms.

15       Accurate HLA DP typing is useful in forensic medicine. For example, it provides evidence as to whether a sample which contains genomic nucleic acids, for example, blood, hair, or semen is derived from a suspect individual. It is also useful in determining an individual's paternity or maternity. The latter is of particular importance in analyzing historical samples.

#### Example 11

##### Probe Hybridization in TMACL

20       When tetramethyl ammonium chloride (TMACL) is present in a hybridization solution, probe discrimination is based on probe length and not on the G, C, A, or T composition of the probe. Thus, by using TMACL in the hybridization solution, one can hybridize and wash many different probes at a single temperature.

25       A suitable hybridization solution for this purpose contains 3 M TMACL; 0.5% SDS; 10 mM Tris-HCl, pH = 7.5; and 0.1 mM EDTA. Hybridizations are carried out at 55°C for 30 to 60 minutes for 19-mer probes DB27, DB28, DB29, DB35, DB34, DB37, DB38, and DB62; at 50°C for 17-mer probes DB30, DB31, DB33, and DB59; and at 60°C for DB40 and DB41. The wash solution is 3 M TMACL; 50 mM Tris-HCl, pH = 8; and 2  
30       mM EDTA. The wash is carried out first at 37°C for 20 minutes, then at the higher stringency temperature (the hybridization temperature) for 10 minutes. Detection of hybridization is carried out as described in Example 10.

Additional probes useful for this, or any other, hybridization format for purposes of the present invention are shown below (X is HRP).

	<u>Probe Designation</u>	<u>Sequence</u>	<u>Length</u>	<u>Amino Acid Sequence</u>
5	DB70	X-GAATTACCTTTTCCAGGGAC	20-MER	LFQG
	DB92	X-GACCTCCTGGAGGAGGAGC	19-MER	L-E
	DB93	X-GACCTCCTGGAGGAGAGGC	9-MER	L-R
	DB94	X-AGCTGGGCGGGCCCATGAC	19-MER	GGPM
	DB95	X-AGCTGGACGAGGCCGTGAC	19-MER	DEAV
10	DB64	X-CTGGTCGGGCCCCATGACC	18-MER	VGPM
	DB72	X-ACATCCTGGAGGAGAAGC	18-MER	I-K
	DB73	X-ACATCCTGGAGGAGGAGC	18-MER	I-E
	DB74	X-ACCTCCTGGAGGAGAAGC	18-MER	L-K
	DB71	X-TTACGTGTACCTGGGAC	17-MER	VYQG
15	DB75	X-CCTGATGAGGAGTACTG	17-MER	DEE
	DB76	X-CTGGGCGGGCCCATG	15-MER	GGPM
	DB77	X-CTGGACGAGGCCGTG	15-MER	DEAV



We Claim:

1. A process for determining an individual's HLA DP genotype from a nucleic acid containing sample obtained from the individual comprising:

(a) amplifying a target region of the nucleic acids in the sample, which target region contains a polymorphic region of an HLA DP gene;

(b) mixing the amplified nucleic acids with a panel of sequence specific oligonucleotide (SSO) probes for variant segments of HLA DP genes under conditions wherein SSO probes bind to said amplified nucleic acids to form stable hybrid duplexes only if they are exactly complementary; and

(c) detecting hybrids formed between the amplified nucleic acids and the SSO probes.

2. The process of Claim 1, wherein the target region amplified is a sequence from exon two of a DPbeta gene, and the panel of SSO probes are complementary to variant segments of said exon of the DPbeta gene.

3. The process of Claim 1, wherein amplification is of exon two of a DPalpha gene, and wherein the SSO probes are complementary to variant segments of said exons.

4. The process of Claim 1, wherein the amplifying comprises treating said sample under conditions suitable for carrying out a polymerase chain reaction.

5. The process of Claim 1, wherein the panel of SSO probes comprises probes to allelic variants of at least one variable segment of DPbeta, wherein each probe is comprised of a nucleotide sequence which is complementary to a variant sequence of a variable segment of DPbeta that is 17 to 23 nucleotides in length.

6. The process of Claim 4, wherein said polymerase chain reaction is carried out in the presence of a primer selected from the group consisting of GH98, GH99, DB01, DB03, UG19, and UG21 primers.

7. The process of Claim 5, wherein the probes of the panel are comprised of nucleotide sequences capable of hybridizing to a polymorphic DNA sequence of a second exon of a DPbeta allele, wherein said polymorphic sequence is selected from the group consisting of sequences specific for the polymorphic regions centered about codons 8-11, 36, 55-57, 65-69, 76, and 84-87.

5 8. The process of Claim 6, wherein the probes of the panel are selected from the group consisting of:

5'GAATTACCTTTTCCAGGGA; 5'ATTACGTGTACCAGTTACG;  
5'ATTACGTGTACCAGTTA; 5'CGTAACTGGTACACGTAAT;  
5'CGTCCCTGGTACACGTAAT; 5'ATTACGTGCACCAGTTACG;  
10 5'CCTGCTGCGGAGTACTG; 5'CAGTACTCCTCATCAGG;  
5'CAGTACTCCGCCTCAGG; 5'CCTGAGGCGGAGTACTG;  
5'CCTGATGAGGACTACTG; 5'GACATCCTGGAGGAGAAGC;  
5'GCTCCTCCTCCAGGATGTC; 5'GACCTCCTGGAGGAGAAGC;  
5'GCTCCTCCTCCAGGAGGTC; 5'GACCTCCTGGAGGAGGAG;  
15 5'GACCTCCTGGAGGAGAGG; 5'CTGCAGGGTTCATGGGCCCCCG;  
5'CTGCAGGGTTCACGGCCTCGTC; 5'GAATTACCTTTTCCAGGGAC;  
5'GACCTCCTGGAGGAGGAGC; 5'GACCTCCTGGAGGAGAGGC;  
5'AGCTGGGCGGGCCCATGAC; 5'AGCTGGACGAGGCCGTGAC;  
5'CTGGTCCGGGCCCATGACC; 5'ACATCCTGGAGGAGAAGC;  
20 5'ACATCCTGGAGGAGGAGC; 5'ACCTCCTGGAGGAGAAGC;  
5'TTACGTGTACCTGGGAC; 5'CCTGATGAGGAGTACTG;  
5'CTGGGCGGGCCCATG; and 5'CTGGACGAGGCCGTG.

9. An oligonucleotide probe selected from the group consisting of:

5'GAATTACCTTTTCCAGGGA; 5'ATTACGTGTACCAGTTACG;  
25 5'ATTACGTGTACCAGTTA; 5'CGTAACTGGTACACGTAAT;  
5'CGTCCCTGGTACACGTAAT; 5'ATTACGTGCACCAGTTACG;  
5'CCTGCTGCGGAGTACTG; 5'CAGTACTCCTCATCAGG;  
5'CAGTACTCCGCCTCAGG; 5'CCTGAGGCGGAGTACTG;  
5'CCTGATGAGGACTACTG; 5'GACATCCTGGAGGAGAAGC;  
30 5'GCTCCTCCTCCAGGATGTC; 5'GACCTCCTGGAGGAGAAGC;

5'GCTCCTCCTCCAGGAGGTC; 5'GACCTCCTGGAGGAGGAG;  
5'GACCTCCTGGAGGAGAGG; 5'CTGCAGGGTCATGGGCCCCCG;  
5'CTGCAGGGTCACGGCCTCGTC; 5'GAATTACCTTTTCCAGGGAC;  
5'GACCTCCTGGAGGAGGAGC; 5'GACCTCCTGGAGGAGAGGC;  
5'AGCTGGGCGGGCCCATGAC; 5'AGCTGGACGAGGCCGTGAC;  
5'CTGGTCGGGCCCCATGACC; 5'ACATCCTGGAGGAGAAGC;  
5'ACATCCTGGAGGAGGAGC; 5'ACCTCCTGGAGGAGAAGC;  
5'TTACGTGTACCTGGGAC; 5'CCTGATGAGGAGTACTG;  
5'CTGGGCGGGCCCATG; and 5'CTGGACGAGGCCGTG.

10. A primer selected from the group consisting of DB01, DB03, GH98, GH99, UG19, and UG21 primers.

11. SSO probes useful for determining an individual's HLA DP genotype from a nucleic acid containing sample obtained from the individual, said probes comprising a nucleotide sequence that is totally complementary to a variant sequence of a variable segment of the second exon of a DPbeta allele and is 17 to 23 nucleotides in length.

12. SSO probes which are useful for determining an individual's HLA DP genotype from a nucleic acid containing sample obtained from the individual, said probes being comprised of a nucleotide sequence which is totally complementary to a variant sequence of a variable segment of the second exon of a DPalpha allele and is 17 to 23 nucleotides in length.

13. A kit useful for determining an individual's HLA DP genotype comprising:  
(a) a panel of SSO probes for allelic variant sequences in said target region; and  
(b) instructions for determining the genotype by utilizing kit ingredients.

14. The kit of Claim 13 further comprising a container containing oligonucleotide primers useful for amplification of a target region of an HLA DP gene.

15. A process for determining an individual's susceptibility to an autoimmune disease comprising determining the individual's HLA DP genotype according to the process of Claim 1 and determining whether the individual's genotype is one which is linked to an autoimmune disease.

5 16. The process of Claim 15, wherein the autoimmune disease is juvenile rheumatoid arthritis, and the individual's HLA DP genotype comprises the DPB2.1 allele.

17. The process of Claim 15, wherein the autoimmune disease is IDDM.

10 18. The process of Claim 15, wherein the autoimmune disease is CD and the genotype is positive for an allele selected from the group consisting of DPB13, DPB1, DPB3, and DPB4.2.

15 19. A process of providing forensic evidence concerning the derivation of a sample which contains genomic nucleic acids comprising determining according to the process in Claim 1 the HLA DP of the sample and of the suspected individual, comparing the HLA DP of the individual and of the sample, and deducing whether the sample is derived from the individual.

## METHOD FOR HLA DP TYPING

This invention relates to a method and compositions for determining the HLA DP genotype of an individual. In a preferred embodiment, the invention relates to using gene amplification methodology disclosed and claimed in U.S. Patent Nos. 4,683,195 and 4,683,202 and the dot-blot and allele-specific oligonucleotide probe technology as disclosed and claimed in U.S. Patent 4,683,194. The methods and probes of the invention specifically relate to the detection of the polymorphic class II HLA DP genes. The invention relates to the fields of molecular biology, diagnostic medicine, and forensics.

The class II loci of the human major histocompatibility complex encode the HLA D cell surface glycoproteins which are expressed on B lymphocytes, activated T lymphocytes, macrophages, and dendritic cells. These proteins, which are individually designated DR, DQ, and DP, are composed of an alpha and a highly polymorphic beta subunit and are responsible for the presentation of antigen to T cells. The variability in the highly polymorphic beta subunit is localized to the amino-terminal extracellular domain, which is thought to interact with the T cell receptor and antigen peptide fragments. The genes encoding the class II HLA proteins are located on the short of chromosome six in humans. The genes encoding the HLA DPalpha and DPbeta chains are clustered at the centromeric end of this region and, perhaps due to low levels of expression, were the last of the HLA D genes to be discovered and are, therefore, the least well characterized. The structures, sequences, and polymorphisms in the HLA D region have been reviewed in Trowsdale et al., 1985, *Immunol. Rev.* 85:5-43, incorporated herein by reference.

The polymorphism of the HLA D region gene products has, in general, been defined by serologic typing reagents and by the mixed lymphocyte culture (MLC) reaction in which T cell proliferation in response to homozygous typing cells (HTC) is measured in culture. The HLA DP antigens were originally defined by their ability to stimulate a strong secondary response in specifically primed T cells, a method known as primed lymphocyte typing (PLT) and described by Mawas et al., 1981, *Tissue Antigens* 15:458-466; Want et al., 1978, *Immunogenetics* 6:107-115; and Shaw et al., 1980, *J. Exp. Med.* 152:565-580. The HLA DP antigens elicit only a weak response in a primary MLC, and unlike the studies of HLA DR and HLA DQ polymorphism, the analysis of allelic variation in the HLA DP region has been complicated by the lack of availability of serologic reagents and of typing

cells. In addition, the specific cell lines used in the PLT assay are difficult to generate, and the typing assay is slow and somewhat variable from laboratory to laboratory.

Cellular (see Odum *et al.*, 1987, Tissue Antigens 29:101-109), biochemical (see Londeau *et al.*, 1987, Immunogenetics 25:403-407), and restriction fragment length polymorphism (RFLP, see Hyldig-Nielsen *et al.*, 1987, Proc. Natl. Acad. Sci. USA 84:1644-1648) analyses have indicated that the degree of polymorphism in the DP region may be more extensive than the currently serologically, immunologically, or PLT-defined DPw1 through DPw6 types. The RFLP approach showed that the RFLP of the DP region was relatively extensive and that some of the fragments were strongly associated with certain DP antigens. However, the RFLP technique has certain limitations. An allele carrying a variant sequence is identifiable only if the variant nucleotide is within the recognition site of a restriction enzyme used in the analysis or if a polymorphic restriction site is in linkage disequilibrium with a specific coding sequence variation. In addition, RFLP analysis simply provides evidence that a coding sequence variation exists but does not provide information on the exact nature of the variation. Moreover, relatively large fragments of the genomic nucleic acid must be used for the analysis. This latter requirement often rules out the use of samples which have been kept under conditions which result in the degradation of the genomic DNA.

Accurate DP typing may prove important in several medical applications. Genetic recombination between the DP loci and the serologically typed DR loci can occur such that serologically identical sibs may not be matched at DP. The HLA DP differences have been revealed by RFLP analysis in several cases of acute graft vs. host disease between apparently HLA-identical donor and recipient pairs, as described by Amar *et al.*, 1987, J. Immunology 138:1947-1953. Furthermore, several autoimmune diseases, including coeliac disease, have been shown to be associated with specific DP types, defined either by PLT, Odum *et al.*, 1986, Tissue Antigens 28:245-250, or by RFLP, Howell *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:222-226, analysis.

A significant improvement in DNA amplification, the polymerase chain reaction (PCR) technique, was disclosed by Mullis in U.S. Patent No. 4,683,195, and methods for utilizing PCR in the cloning and detection of nucleic acids were disclosed by Mullis *et al.* in U.S. Patent No. 4,683,202, both of which patent disclosures are hereby incorporated herein by reference. In the PCR technique, short oligonucleotide primers are prepared to match opposite ends of the sequence to be amplified. The sequence between the primers need not be known. A sample of nucleic acid (DNA or RNA, although RNA is first

converted to cDNA in the PCR process) is extracted and denatured, preferably by heat, and hybridized with oligonucleotide primers which are present in molar excess. Polymerization is catalyzed by a polymerase in the presence of deoxynucleotide triphosphates (dNTPs). This results in two "long products" which contain the respective primers at their 5'-termini, covalently linked to the newly synthesized complements of the original strands. The replicated DNA is again denatured, hybridized with oligonucleotide primers, returned to polymerizing conditions, and a second cycle of replication is initiated. The second cycle provides the two original strands, the two long products from cycle 1 and two long products of cycle 2, and two "short products" replicated from the long products produced in cycle 1. The short products contain sequences (sense or antisense) derived from the target sequence and flanked at the 5' end with a primer and at the 3'-end with a sequence complementary to a primer. On each additional cycle, the number of short products is replicated exponentially. Thus, the PCR process causes the amplification of a specific target sequence and allows for the detection of sequences initially present in a sample in only extremely small amounts.

Allelic sequence variations in the gene encoding beta-globin of hemoglobin and in the gene encoding HLA DQalpha have been detected by utilizing allele-specific oligonucleotides (ASO), which will only anneal to sequences that match them perfectly, as described by Saiki et al., 1986, *Nature* 324:163-166. These studies also utilized the PCR procedure to amplify the DNA sequences present in the samples and a dot-blot technique to detect probe hybridization to the sample.

The present invention solves the above listed problems in HLA DP typing and provides a relatively rapid, convenient, practical, accurate, and reproducible method for determining HLA DP genotypes. The method is based in part upon the finding that there are a large number of previously unreported DPbeta alleles. The variation between alleles is of a highly dispersed nature. The present invention also provides nucleic acid probes for the regions of the HLA DPbeta gene that are most informative in discriminating between different DP alleles for use in the method.

These oligonucleotide probes are called SSOs (sequence specific oligonucleotides), which, under the appropriate conditions, are able to bind specifically to their complementary sequences. If a particular probe can be used to identify uniquely an allele, the probe is called an allele specific oligonucleotide (ASO). Because of the dispersed nature of the variation between the DPbeta alleles, rarely is any one probe able to identify uniquely a specific DPbeta allele. Rather, according to the methods of the present

invention, the identity of an allele is inferred from the pattern of binding of a panel of probes, with each individual probe of the panel specific for different segments of the HLA DP gene. The probes comprise a nucleotide sequence totally complementary to a variant sequence of a variable segment of a DP gene. The complementary sequence of the probes is usually from 10 to 30 nucleotides in length, most often 17 to 19 nucleotides in length.

As noted above, PCR is quite an important tool in nucleic acid based diagnostic methods. The novel probes of the invention can of course be used to detect specific sequences in PCR-amplified DNA. To enable practice of PCR-based, HLA DP DNA typing, the present invention provides oligonucleotide primers that allow for the amplification of informative DP regions via PCR.

Accordingly, one aspect of the invention is a process for determining an individual's HLA DP genotype from a nucleic acid containing sample obtained from the individual comprising: (a) amplifying a target region of said nucleic acid which contains a polymorphic region of an HLA DP gene; (b) hybridizing the amplified nucleic acid with a panel of sequence specific oligonucleotide (SSO) probes specific for variant segments of HLA DP genes under conditions that allow said SSO probes and amplified nucleic acids to form stable hybrid duplexes; and (c) detecting hybrids formed between the amplified nucleic acids and the SSO probes.

Another aspect of the invention relates to kits useful for determining the HLA DP genotype of an individual; these kits comprise a panel of SSO probes for allelic variant sequences in said target region; and (b) instructions for determining the genotype by utilizing kit ingredients.

To aid in understanding the invention, several terms are defined below.

"Genotype" refers to a description of the genotypic variants of an allele present in an individual (or sample). The genotype of an individual may be determined, for example, by the use of serologic reagents, by the use of typing cells, by RFLP analysis of the genome, and, as discussed below, by the use of SSOs.

"HLA DP region" or "DP" refers to that region of the HLA D complex, described in Trowsdale *et al.*, *supra*, which lies on the centromeric side of the HLA D region and contains two alpha and two beta genes, one pair of which are pseudogenes.

"Oligonucleotide" refers to primers, probes, nucleic acid fragments to be detected, nucleic acid controls, and unlabeled blocking oligomers and is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides. The exact size of an oligonucleotide will depend upon many factors and the ultimate function or use of the



oligonucleotide. Oligonucleotides can be prepared by any suitable method. Such methods include, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphotriester method of Narang *et al.*, 1979, *Meth. Enzymol.* 68:90; the phosphodiester method of Brown *et al.*, 1979, *Meth. Enzymol.* 68:109; the diethylphosphoramidite method of Beaucage *et al.*, 1981, *Tetrahedron Lett.* 22:1859-1862; and the solid support method in U.S. Patent No. 4,458,066.

"Polymorphic" or "DNA polymorphism" refers to the condition in which two or more variations of a specific DNA sequence coexist in the same interbreeding population.

"Primer" refers to an oligonucleotide, whether natural or synthetic, capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, i.e., in the presence of four different nucleotide triphosphates and an agent for polymerization (i.e., DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably an oligodeoxyribonucleotide and is single stranded for maximum efficiency in amplification, but may also be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The exact length of a primer will depend on many factors, but typically ranges from 15 to 25 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template. An example of a non-complementary sequence which may be incorporated into the primer is a sequence which encodes a restriction enzyme recognition site (see U.S. Patent No. 4,800,159).

"Primer," as used herein, may refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding one or both ends of the target region to be amplified. For instance, if a nucleic acid sequence is inferred from a protein sequence, a "primer" is actually a collection of primer oligonucleotides containing sequences representing all possible codon variations based on the degeneracy of the genetic code. One of the primer oligonucleotides in this collection will be homologous with the end of the target sequence. Likewise, if a "conserved" region shows significant levels of polymorphism in a population, mixtures of primers can be prepared that will amplify adjacent sequences. A primer can be labeled, if desired, by incorporating a label detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include  $^{32}\text{P}$ , fluorescent dyes, electron-dense reagents, enzymes (as

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commonly used in ELISAS), biotin, or haptens or proteins for which antisera or monoclonal antibodies are available. A labels can also be used to "capture" the primer, so as to facilitate the immobilization of either the primer or amplified DNA on a solid support.

5 "Restriction endonucleases" and "restriction enzymes" refer to enzymes, usually of bacterial origin, that cut double-stranded DNA at or near a specific nucleotide sequence.

"Restriction fragment length polymorphism" or "RFLP" refers to the differences in DNA nucleotide sequences that are randomly distributed throughout the entire genome and that produce different restriction endonuclease patterns for different individuals upon  
10 digestion of genomic DNA.

"Sequence-specific oligonucleotide" or "SSO" refers to oligonucleotides that have an exactly complementary sequence to the sequence to be detected, typically sequences characteristic of a particular DP allele, which under "sequence-specific hybridization conditions will hybridize only to that exact complementary target sequence. The  
15 hybridization is under stringent conditions. Stringent hybridization conditions are known in the art, and are described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual (New York, Cold Spring Harbor Laboratory, 1982). Depending on the sequences being analyzed, one or more sequence-specific oligonucleotides may be employed for each sequence. The terms "probe" and "SSO probe" are used  
20 interchangeably with SSO.

"Target region" refers to a region of a nucleic acid which is to be analyzed, which usually contains polymorphic DNA sequences.

"Thermostable polymerase enzyme" refers to an enzyme which is relatively stable to heat and which catalyzes the polymerization of nucleotides to form primer extension  
25 products that are complementary to one of the nucleic acid strands of the target sequence. Generally, the enzyme will initiate synthesis at the 3'-end of the target sequence utilizing the primer and will proceed in the 5'-direction along the template until synthesis terminates. A purified thermostable polymerase enzyme is described more fully in European Patent Office (EPO) Publication 258,017, incorporated herein by reference, and  
30 is commercially available from Perkin-Elmer Cetus Instruments.

#### Disclosure of the Invention

According to a first embodiment of this invention there is provided a process for determining an individual's HLA DPbeta genotype from a nucleic acid containing sample obtained from the individual comprising:

35 a) amplifying a sequence from exon two of a DPbeta gene in the nucleic acids in the sample;

b) mixing the amplified nucleic acids with a panel of sequence specific oligonucleotide (SSO) probes, wherein each probe comprises a nucleotide sequence complementary to a variant sequence of a variable segment of said HLA DPbeta gene

wherein the variant region is selected from the group consisting of the variable segments located at codons 8-11, 36, 55-57, 65-69, 76 and 84-87, under conditions wherein SSO probes bind to said amplified nucleic acids to form stable hybrid duplexes only if they are exactly complementary; and

- 5 c) detecting hybrids formed between the amplified nucleic acids and the SSO probes.

According to a second embodiment of this invention there is provided an oligonucleotide probe selected from the group consisting of:

5'GAATTACCTTTTCCAGGGA; 5'ATTACGTGTACCAGTTACG;  
5'ATTACGTGTACCAGTTA; 5'CGTAACTGGTACACGTAAT;  
5'CGTCCCTGGTACACGTAAT; 5'ATTACGTGCACCAGTTACG;  
5'CCTGCTGCGGAGTACTG; 5'CAGTACTCCTCATCAGG;  
5'CAGTACTCCGCCTCAGG; 5'CCTGAGGCGGAGTACTG;  
5'CCTGATGAAGACTACTG; 5'GACATCCTGGAGGAGAAGC;  
5'GCTCCTCCTCCAGGATGTC; 5'GACCTCCTGGAGGAGAAGC;  
5'GCTCCTCCTCCAGGAGGTC; 5'GACCTCCTGGAGGAGGAG;  
5'GACCTCCTGGAGGAGAGG; 5'CTGCAGGGTCATGGGCCCCCG;  
5'CTGCAGGGTCACGGCCTCGTC; 5'GAATTACCTTTTCCAGGGAC;  
5'GACCTCCTGGAGGAGGAGC; 5'GACCTCCTGGAGGAGAGGC;  
5'AGCTGGGCGGGCCCATGAC; 5'AGCTGGACGAGGCCGTGAC;  
5'CTGGTCGGGCCCCATGACC; 5'ACATCCTGGAGGAGAAGC;  
5'ACATCCTGGAGGAGGAGC; 5'ACCTCCTGGAGGAGAAGC;  
5'TTACGTGTACCTGGGAC; 5'CCTGATGAGGAGTACTG;  
5'CTGGGCGGGCCCATG; and 5'CTGGACGAGGCCGTG.

- 10 According to a third embodiment of this invention there is provided a primer selected from the group consisting of DB01, DB03, UG19 and UG21 primers.

According to a fourth embodiment of this invention there is provided sequence specific oligonucleotide (SSO) probes useful for determining an individual's HLA DPbeta  
15 genotype from a nucleic acid containing sample obtained from the individual, each of said probes comprising a nucleotide sequence that is totally complementary to a variant sequence of a variable segment selected from the group consisting of the variable segments located at codons 8-11, 36, 55-57, 65-69, 76 and 84-87 of the second exon of a DPbeta allele.

- 20 According to a fifth embodiment of this invention there is provided a kit useful for determining an individual's HLA DP genotype comprising a panel of SSO probes for allelic variant sequences in said target region according to this invention.

According to a sixth embodiment of this invention there is provided a process for  
25 determining an individual's susceptibility to an autoimmune disease comprising determining the individual's HLA DPbeta genotype according to the process of this



genes, SSO probes are provided for the detection of the variant genotypes. The variations between the different DP alleles are dispersed. Therefore, one probe alone is rarely able to identify uniquely a specific DPbeta allele. Rather, the identity of an allele is inferred from the pattern of binding of a panel of probes, with each individual probe of the panel specific to different segments of a DP gene.

In a preferred embodiment of the invention, the process for DNA based typing of HLA DP genotypes is comprised of amplifying a nucleic acid sequence which contains a variable portion of an HLA DP gene, determining the variant HLA DP sequence present using SSO probes; and inferring the HLA DP genotype from the pattern of binding of the SSO probes to the amplified target sequence. To facilitate practice of this preferred embodiment, the present invention provides primers useful in amplifying by PCR the HLA DP target region.

In this preferred method, a sample containing nucleic acid is obtained from an individual whose HLA DP genotype is to be determined. Any type of tissue containing HLA DP nucleic acid may be used for purposes of the present invention. Because the present invention also is compatible with amplified nucleic acids, and because the PCR technique can amplify extremely small quantities of nucleic acid, samples containing vanishingly small amounts of nucleic acid can be typed for the presence of particular HLA DP variants by the method of the present invention. For instance, even a single hair, contains enough DNA for purposes of the present invention, as evidenced by the work with DQalpha described by Higuchi et al., 1988, *Nature* 332:543-546.

In general, the nucleic acid in the sample will be DNA, most usually genomic DNA. However, the present invention can also be practiced with other nucleic acids, such as messenger RNA or cloned DNA, and the nucleic acid may be either single-stranded or double-stranded in the sample and still be suitable for purposes of the present invention. Those of skill in the art recognize that whatever the nature of the nucleic acid, the nucleic acid can be typed by the present method merely by taking appropriate steps at the relevant stage of the process. If PCR is used to amplify the nucleic acid in the sample, then the sample will usually comprise double-stranded DNA when typed with the novel probes of the invention.

As noted above, in a preferred embodiment, the HLA DP typing method and probes of the invention are used in conjunction with PCR-amplified target DNA. Those practicing the present invention should note, however, that amplification of HLA DP target sequences in a sample may be accomplished by any known method which provides sufficient

amplification so that the target sequence may be detected by nucleic acid hybridization to an SSO probe. Although the PCR process is well known in the art (see U.S. Patent Nos. 4,683,195 and 4,683,202) and although a variety of commercial vendors, such as Perkin-Elmer/Cetus Instruments, sell PCR reagents and publish PCR protocols, some general PCR information is provided below for purposes of clarity and full understanding of the invention to those unfamiliar with the PCR process.

To amplify a target nucleic acid sequence in a sample by PCR, the sequence must be accessible to the components of the amplification system. In general, this accessibility is ensured by isolating the nucleic acids from the sample. A variety of techniques for extracting nucleic acids from biological samples are known in the art. For example, see those described in Maniatis *et al.*, Molecular Cloning: A Laboratory Manual, (New York, Cold Spring Harbor Laboratory, 1982). Alternatively, if the sample is fairly readily disruptable, the nucleic acid need not be purified prior to amplification by the PCR technique, i.e., if the sample is comprised of cells, particularly peripheral blood lymphocytes or amniocytes, lysis and dispersion of the intracellular components may be accomplished merely by suspending the cells in hypotonic buffer.

Because the nucleic acid in the sample is first denatured (assuming the sample nucleic acid is double-stranded) to begin the PCR process, and because simply heating some samples results in the disruption of cells, isolation of nucleic acid from the sample can sometimes be accomplished in conjunction with strand separation. Strand separation can be accomplished by any suitable denaturing method, however, including physical, chemical, or enzymatic means. Typical heat denaturation involves temperatures ranging from about 80°C to 105°C for times ranging from about 1 to 10 minutes. Strand separation may also be induced by a helicase, an enzyme capable of exhibiting helicase activity. For example, the enzyme RecA has helicase activity in the presence of ATP. The reaction conditions suitable for strand separation by helicases are known in the art (see Kuhn Hoffman-Berling, 1978, CSH-Quantitative Biology 43:63 and Radding, 1982, Ann. Rev. Genetics 16:405-436).

As noted above strand separation may be accomplished in conjunction with the isolation of the sample nucleic acid or as a separate step. In addition, strand separation can be achieved simultaneously with the next step in the PCR process: annealing of primers and synthesis of primer extension products. In this embodiment of the PCR process, the temperature is very carefully controlled so that strand separation and primer annealing and extension occur in equilibrium. In this embodiment of the PCR process, the reaction is

5 catalyzed by a heat-stable polymerase and carried out at an elevated temperature. The temperature is one at which the enzyme is thermostable, and at which the nucleic acids are in an equilibrium of single and double strands, so that sufficient primer will anneal to template strands to allow a reasonable rate of polymerization. In the preferred embodiment of the PCR process, however, strand separation is achieved by heating the reaction to a sufficiently high temperature for an effective time to cause the denaturation of the duplex, but not to cause an irreversible denaturation of the polymerase (see EP No. 258,017).

10 No matter how strand separation is achieved, however, once the strands are separated, the next step in PCR involves hybridizing the separated strands with primers that flank the target sequence. The primers are then extended to form complementary copies of the target strands, and the cycle of denaturation, hybridization, and extension is repeated as many times as necessary to obtain the desired amount of amplified nucleic acid.

15 As noted above, the present invention provides PCR primers for HLA DP DNA amplification and typing. These primers are complementary to sequences in the conserved regions that flank the target sequences in the variant regions of the HLA DP loci. For purposes of the present invention, the preferred variant region of the HLA DP loci is the second exon of the DPalpha and DPbeta genes. For successful PCR amplification, the present primers are designed so that the position at which each primer hybridizes along a duplex sequence is such that an extension product synthesized from one primer, when it is separated from its template (complement), serves as a template for the extension of the other primer to yield an amplified segment of nucleic acid of defined length. Moreover, primers are provided that will bind preferentially to the HLA DP region under selective annealing conditions.

25 Template-dependent extension of primers in PCR is catalyzed by a polymerizing agent in the presence of adequate amounts of four deoxyribonucleotide triphosphates (dATP, dGTP, dCTP, and dTTP) in a reaction medium comprised of the appropriate salts, metal cations, and pH buffering system. Suitable polymerizing agents are enzymes known to catalyze template-dependent DNA synthesis. For example, if the template is RNA, a suitable polymerizing agent to convert the RNA into a complementary DNA (cDNA) sequence is reverse transcriptase (RT), such as avian myeloblastosis virus RT. Once the target for amplification is DNA, suitable polymerases include, for example, *E. coli* DNA polymerase I or its Klenow fragment, T<sub>4</sub> DNA polymerase, and Taq polymerase, a heat stable DNA polymerase isolated from *Thermus aquaticus* and commercially available from Perkin-Elmer/Cetus Instruments (PECI). The latter enzyme is widely used in the

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amplification and sequencing of nucleic acids. The reaction conditions for using DNA polymerases are known in the art, and are described in, for example, the treatise Methods in Enzymology, and in Maniatis et al., Molecular Cloning: A Laboratory Manual, supra.

5 The PCR method can be performed in a step-wise fashion, where after each step new reagents are added, or in a fashion where all of the reagents are added simultaneously, or in a partial step-wise fashion, where fresh or different reagents are added after a given number of steps. For example, if strand separation is induced by heat, and the polymerase is heat-sensitive, then the polymerase will have to be added after every round of strand separation. However, if, for example, a helicase is used for denaturation, or if a  
10 thermostable polymerase is used for extension, then all of the reagents may be added initially, or, alternatively, if molar ratios of reagents are of consequence to the reaction, the reagents may be replenished periodically as they are depleted by the synthetic reaction.

Those skilled in the art will know that the PCR process is most usually carried out as an automated process with a thermostable enzyme. In this process, the reaction mixture is  
15 cycled through a denaturing region, a primer annealing region, and a reaction region. A machine specifically adapted for use with a thermostable enzyme is disclosed more completely in EP 236,069 and is commercially available from PECL.

One reason, as noted above, the PCR process is important in the method of the present invention is that the PCR process can be used to amplify the sample nucleic acid prior to HLA DP DNA typing. Another important use of PCR for purposes of the present  
20 invention, however is for determining the nucleotide sequence of previously undiscovered allelic variants which exist in the HLA DP region, so that probes for those variants can be constructed and used in the present method. In this use of the PCR process, polymorphic regions of the DPalpha and DPbeta genes are amplified, and the nucleotide sequences of these polymorphic target regions, for example, the second exon of the DPalpha and DPbeta  
25 genes, are determined. As illustrated below, it is also useful for the cells containing a particular variant to be typed by serological typing, mixed lymphocyte typing, or primed lymphocyte typing to correlate the nucleotide sequence of a particular variant with the DP type established by prior art methods.

30 Analysis of the nucleotide sequence of the target region of a DP variant allele can be readily performed by direct analysis of the PCR products. A preferred sequencing protocol is described by Innis et al., 1988, Proc. Natl. Acad. Sci. 85:9436-9440, incorporated herein by reference. A process for direct sequence analysis of PCR amplified products is also described by Saiki et al., 1988, Science 239:487-491. Alternatively, the amplified



target sequence may be cloned prior to sequence analysis, as described by Scharf *et al.*, 1986, *Science* 233:1076-1078.

As discussed in the Examples below, a panel of DPw typed cells, representing a large number of different haplotypes, have been analyzed by PCR and nucleotide sequencing of the second exon of the DPbeta gene. As a result of this effort and similar efforts using samples obtained from a variety of individuals suffering from autoimmune diseases, discussed in greater detail below, 22 different allelic variants in this locus were discovered. In general, the results demonstrated that specific DPbeta sequences correlate with the standard PLT-defined DPw1 through DPw6 specificities. The rare exceptions may reflect the difficulty of obtaining a standard and reproducible PLT DPw typing system, which difficulties highlight the advantages of the present method. In this regard, it is relevant that the cell line Cox, originally typed as DPw1, has recently been retyped as DPw3 and contains the DPB3 allele.

Other interesting and important correlations between the serologically defined DP types and DP variant nucleotide sequences have been discovered as a result of the advances provided by the present invention. For instance, the DPw4 type of the prior art can now be subdivided into two DPbeta subtypes (designated DPB4.1 and DPB4.2) by the method of the present invention. The DPB4.2 allele was found in two (APD and LBI) DPw4 homozygous typing cells (HTCs) known to have an unusual DPw4 typing. DPbeta subtypes (designated DPB2.1 and DPB2.2) for the DPw2 type were also discovered as a result of the present invention.

Yet another significant advance provided by the present invention relates to the discovery of a large number of variant DPbeta alleles in a variety of cells that type by the prior art methods as DP "blank." These variant alleles have been designated with DPB numbers of 7 and above. Further analysis of DPw blank haplotypes will probably lead to the identification and characterization of additional DPbeta alleles that can then be typed by the present method. DPw blank cells are those which fail to stimulate the existing PLT panels of T cell reagents. Unlike the serologic DQw blank specificity, which, as described by Horn *et al.*, 1988, *Proc. Natl. Acad. Sci. USA* 85:6012-6016, is encoded by a unique DQB sequence, the DPw blank specificity is heterogeneous. Because the haplotypic frequency of the DPw blank is estimated to be 40%, the present invention is especially important in that it for the first time enables the subtyping of this large DPw type.

The availability of DPbeta DNA sequences from DPw typed cells not only makes possible the subdivision of the serological DPw types but also makes possible the

identification of the linear epitopes defined by serologic and cellular typing reagents. For example, the antibody SP42 reacts with DPw2 and DPw4 cells. The only sequence unique to these two specificities is the GlyGlyProMet (GGPM) at positions 84 to 87, which are therefore believed to constitute the SP42 epitope. Likewise, the antibody SP3 reacts with DPw3 and DPw6 cells, as well as with some additional cells like the DPw blank lines Tok and Akiba. These lines bear the specificity Cp62 and contain the closely related DPbeta alleles DPB9 and DPB12. The only sequence unique to SP3 reactive cells is the acidic AspGluAsp (DED) sequence at positions 55 to 57. The SP3 epitope therefore seems to map to this region of the DPbeta chain.

As yet another example of how the novel DP sequences of the invention allow the mapping of serologically defined epitopes, the antibody DP11.1 reacts with DPw2 and DPw4 cells. Bodmer et al., 1987, Proc. Acad. Sci. USA 84:1644-1648, showed with Western blot analysis that this antibody binds to the DPalpha chain. Given the pattern of DPbeta polymorphisms provided by the present invention, this observation suggests that the DP11.1 antibody binds to a conformational determinant formed by the DPalpha chain and DPbeta chains containing the GlyGlyProMet (GGPM) sequence at positions 84 to 87.

Polymorphic residues in the HLA DP region may also constitute epitopes recognized by T cell clones. Cells from clone 1666 react with DPw5 and some DPw2 cells. ABL (DPB2.2) is positive and WPV (DPB2.1) is negative, as disclosed at the Tenth International Histocompatibility Workshop. These C1666 cells may recognize the GluAlaGlu (EAE) residues at position 55 to 57, because this sequence is unique to the DPB2.2 and DPB5 alleles. A Leu (as opposed to Phe) residue at position 35 is also unique to C1666 reactive cells.

The same approach of correlating specific polymorphic residues with DPw specificities can be used to map the epitopes defined by PLT typing. For example, the only difference between the DPB2.1 and DPB4.2 alleles is the Glu to Lys change at position 69. Because cells containing these alleles are distinguished from each other in the PLT system, the charge of the polymorphic position 69 residue is believed to be involved in the epitope recognized by PLT typing cells. Similarly, the only differences between the DPB3 and DPB6 alleles is the Lys to Glu substitution at position 69, as well as Val to Met change at position 76.

As illustrated by the foregoing, the novel DPbeta sequences provided by the present invention not only provides the basis for a very useful DPbeta DNA typing method but provides much useful information in the interpretation of serological DPbeta typing results.

The present invention also enables one to perform HLA DNA typing not only on the DPbeta locus but also on the DPalpha locus as well. As illustrated below, at the present time, only two variant alleles are known to exist at the second exon of the DPalpha locus (see Trowsdale *et al.*, *supra*). The protein sequences encoded by the second exon of these two alleles (these alleles are designated DPA1 and DPA2) differ from each other by only three amino acids, and these differences do not appear to correlate with any specific PLT-defined type. Thus, the DPalpha variation is much less extensive than predicted by the RFLP variation detected with DPalpha probes (see Hyldig-Nielsen *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84:1644-1648), suggesting that most of the polymorphisms detected in the DPalpha gene by RFLP analysis are either in noncoding sequences or in a nearby DPbeta locus. The observed correlations between DPw types and DPalpha RFLP markers therefore probably reflects linkage disequilibrium between DPbeta sequence variation and the polymorphic restriction sites detected with the DPalpha probe, as does the association between DRalpha RFLP markers and DR specificities (see Steller *et al.*, 1985, *Proc. Natl. Acad. Sci. USA* 82:8100-8104).

The DNA sequences of the DPalpha and DPbeta genes serve as a useful starting point in the design of the sequence specific oligonucleotide probes of the present invention. These probes are designed so that, under stringent hybridization conditions, the probes hybridize specifically only to exactly complementary sequences in variant segments of the DPalpha and DPbeta alleles. These SSO probes may be of any length which spans the variant sequences in a variant region and allows for sequence specific hybridization, but preferably the hybridizing region of the probe is short, in the range of 10 to 30 bases, and more preferably is about 17 to 19 bases in length. For immobilization, the probe may also contain long stretches of poly T which can be fixed to a solid support by irradiation.

The SSO probes of the invention are also designed to hybridize specifically with a particular variant segment of a DP allele and to have destabilizing mismatches with the other variant sequences known for the particular segment. Preferably, the probes are specific for variant DNA segments in the variable second exons of the DPalpha and DPbeta genes, and even more preferably, the probes are specific for DNA segments encoding the residues near positions 8-11, 36, 55-57, 65-69, 76, and 84-87 of the second exon. Oligonucleotide probes which have been designed to hybridize specifically to the second exons of the DPbeta and DPalpha alleles are described in more detail below and in the Examples.

The probes of the invention can be synthesized and labeled using the techniques described above in the discussion of PCR primers. For example, the probe may be labeled

at the 5'-end with  $^{32}\text{P}$  by incubating the probe with  $^{32}\text{P}$ -ATP and kinase. A suitable non-radioactive label for SSO probes is horseradish peroxidase (HRP). Methods for preparing and detecting probes containing this label are described in the Examples below. For additional information on the use of such labeled probes, see U.S. Patent No. 4,789,630; Saiki et al., 1988, *N. Eng. J. Med.* 319:537-541; and Bugawan et al., 1988, *Bio/Technology* 6:943-947, incorporated herein by reference. Useful chromogens include red leuco dye and TMB.

The probes of the invention can be used to identify the allelic sequences present in a sample by determining which of the SSO probes bind to the HLA DP sequences present in the sample. Suitable assay methods for purposes of the present invention to detect hybrids formed between SSO probes and nucleic acid sequences in a sample are known in the art. For example, the detection can be accomplished using a dot blot format, as described in the Examples. In the dot blot format, the unlabeled amplified sample is bound to a membrane, the membrane incubated with labeled probe under suitable hybridization conditions, unhybridized probe removed by washing, and the filter monitored for the presence of bound probe. When multiple samples are analyzed with few probes, a preferred process requires high stringency hybridization and wash conditions, allowing only perfectly matched hybrids to exist.

An alternative method is a "reverse" dot blot format, in which the amplified sequence contains a label. In this format, the unlabeled SSO probes are bound to the membrane and exposed to the labeled sample under appropriately stringent hybridization conditions. Unhybridized labeled sample is then removed by washing under suitably stringent conditions, and the filter is then monitored for the presence of bound sequences. In another version of the "reverse" dot blot format, the SSO probe is labeled, and the sample nucleic acid is unlabeled. After hybridization and washing, the labeled probe, or a labeled fragment of the probe, is released from the membrane and detected to determine if a sequence in the sample hybridized to the labeled oligonucleotide. Release of label can be achieved by digestion with a restriction enzyme that recognizes a restriction site in the duplex hybrid. This procedure, known as oligomer restriction, is described more fully in U.S. Patent No. 4,683,194 and corresponding EP Patent Publication 164,054, incorporated herein by reference.

Whatever the method for determining which DP SSO probes of the invention hybridize to DP sequences in a sample, the central feature of the DP DNA typing method involves the identification of the HLA DP alleles present in the sample by analyzing the

pattern of binding of a panel of SSO probes. Although single probes of the invention can certainly be used to provide useful information, the variation in the DPbeta alleles is dispersed in nature, so rarely is any one probe able to identify uniquely a specific DP variant. Rather, as shown in the Examples, the identity of an allele is inferred from the pattern of binding of a panel of SSO probes, which are specific to different segments of the DPalpha and DPbeta genes.

DNA typing of HLA DP alleles is useful for many different purposes. For example, DPbeta polymorphism is involved in tissue rejection of allografts. Bone marrow transplantation studies revealed that three apparently HLA-identical donor-recipient pairs who exhibited acute graft versus host disease and weak MLC reactivity were, by RFLP analysis, all different in the DP region, as described by Amar *et al.*, *J. Immunology* 138:1947. Thus, useful methods for preventing graft rejection will involve matching the HLA DP types of the donor and recipient by applying the present method of HLA DP DNA typing: inferring the HLA alleles present in donor and host from the pattern of hybridization of SSO probes to DPbeta and/or DPalpha nucleic acid sequences present in samples obtained from both donor and host.

Another important application for the present method of HLA DP DNA typing is in determining an individual's susceptibility to autoimmune diseases linked to certain HLA DP alleles. The association of particular DP alleles with an autoimmune disease can be discovered by using the present method to determine the frequency with which a given allele is present in individuals suffering from the autoimmune disease and comparing that frequency with the frequency determined for healthy individuals of a control group, as has been done with other HLA typing systems. For example, Howell *et al.*, 1988, *Proc. Natl. Acad. Sci. USA* 85:222-226, reported a significant association between coeliac disease (CD) and a pair of HLA DPalpha and HLA DPbeta RFLPs. Such studies often provide the additional benefit of the discovery of previously uncharacterized DP alleles, in both patient and control groups. For instance, the DPB13 through DPB19 alleles, the sequences of which represent an important aspect of the present invention, were discovered in the course of studies involving autoimmune disease patients and control groups.

In just such a fashion, an initial study of four CD patients who were typed by the method of the present invention revealed that the DPB4.2 allele confers CD susceptibility. A more extensive study, based on samples from Italian individuals, also showed that the calculated relative risk (RR) of CD for the DPB4.2 allele is 9.3 and that six of the eleven CD patients typed by the present method carried the DPB3 allele. About 78% of CD

patients examined by the present method carry either the DPB4.2 or the DPB3 alleles, so that the RR for the presence of either of these alleles is 13.5. In yet another study of U.S. individuals with CD, an increase in the DPB1 allele frequency, as well as an increase in the DPB4.2 allele frequency, was observed when compared to the frequency of these alleles in a control group. Such studies also revealed that specific combinations of DQbeta alleles (DQB2), DQalpha alleles (DQA4) and DPbeta alleles (DPB4.2 or DPB3) are associated with increased risk of CD susceptibility.

As shown from the foregoing, the present invention provides important new methods for determining the CD susceptibility of an individual, which methods comprise determining whether an individual carries a DP allele associated with CD susceptibility. As shown herein, such CD susceptibility-conferring alleles include the DPB4.2, DPB3, and DPB1 alleles, and as DPB4.1 is a relatively common allele, there is an increased frequency of the genotype DPB4.1/4.2 in CD susceptible individuals.

As with CD, the present invention provides similar advances with respect to other serious autoimmune diseases. For instance, studies that correlate allelic frequency with disease show that the frequency of specific DPbeta alleles appears to be higher in insulin dependent diabetes mellitus (IDDM) and myasthenia gravis (MG) patients than in otherwise HLA matched controls. For MG, the frequency of DP alleles that code for the sequence DEAV at the 3' end of the second exon, such as DPB3 and DPB5, is increased. In addition, the frequency of the DPB4.1 allele in MG patients (relative to healthy control individuals) is dramatically decreased, suggesting that DPB4.1 may confer protection from MG. For IDDM, the frequency of DPbeta alleles DPB2.1, DPB1, DPB4.1, and DPB13 is increased. Many of the IDDM patients studied that carried the DPB13 allele also carried the HLA alleles B18 and DR3.

The DP typing methods of the present invention have also provided important advances in determining whether an individual is susceptible to certain forms of arthritis. For instance, patients with classical rheumatoid factor positive adult rheumatoid arthritis (ARA) show an increased frequency of the DPB4.2 allele, although more studies must be completed to ensure that this increase is statistically significant. In addition, scientists have long known that the DPw2 serologic type is associated with susceptibility to pauciarticular juvenile rheumatoid arthritis (JRA). As noted above, the present invention has for the first time allowed the serologic DPw2 type to be subdivided into two DPB DNA types, and the disease-susceptibility typing methods of the present invention demonstrate that the known DPw2-associated susceptibility to JRA is more specifically attributable to the DPB2.1

allele. About 55% of the JRA patients examined were positive for DPB2.1, while only 16% of control individuals had this allele, giving a RR of 6.3 for JRA in individuals with the DPB2.1 allele. The association of JRA with the DPB2.1 allele is independent of linkage with previously defined HLA DP region markers, and the significance of this DPB2.1 association with JRA can be more readily appreciated when one considers that the DPB2.1 sequence differs from the DPB4.2 allele (apparently not associated with JRA susceptibility) by only one amino acid at position 69 of the B1 domain. It should be noted, however, that most JRA patients with the DPB2.1 allele also had a disease-associated DR marker such as DRw8, DR5, or DRw6.

Thus, the present invention also provides a method for detecting JRA susceptibility that comprises treating HLA DP genomic DNA of an individual, preferably after amplification, with oligonucleotide probes specific for the DPB2.1 allele and determining if hybridization has occurred. General practice of the invention will, however, utilize the full panel of DPbeta probes. As noted above, the present method will be useful in determining the DP association with a wide variety of autoimmune diseases, not just JRA. This fact can be more readily grasped by considering the Table below, in which the allelic frequency (number of alleles of the indicated DP type divided by total number of DP alleles present and then multiplied by 100) for the various DP alleles in a number of patient groups and a control group is shown. In the Table, MS indicates multiple sclerosis patients.

## HLA-DP ALLELIC FREQUENCIES IN SELECTED PATIENT GROUPS

	Allele	Controls (n=50)	MG (n=23)	JRA (n=44)	MS (n=21)	ARA (n=23)
	1	6%	9%	3%	2%	5%
	2.1	10%	6%	30%	12%	12%
5	2.2	0%	2%	0%	6%	0%
	3	6%	13%	11%	17%	11%
	4.1	56%	25%	40%	48%	41%
	4.2	10%	10%	6%	6%	22%
	5	2%	10%	0%	0%	2%
10	6	2%	0%	2%	0%	2%
	7	0%	0%	0%	2%	0%
	8	0%	6%	0%	0%	0%
	9	0%	6%	1%	0%	0%
	10	1%	6%	1%	0%	2%
15	11	3%	2%	1%	0%	0%
	12	0%	0%	0%	0%	0%
	13	1%	2%	1%	2%	0%
	14	2%	0%	0%	2%	2%
	15	0%	0%	2%	0%	2%
20	16	0%	0%	1%	2%	0%
	17	1%	2%	0%	0%	2%
	18	0%	0%	0%	0%	0%
	19	0%	0%	0%	0%	0%
	20	0%	2%	0%	0%	0%

25 n = number of individuals typed; 2n is the number of chromosomes tested; controls were individuals from Northern California.

30 In general, these studies of autoimmune disease suggest that the change of polymorphic residues at positions 55, 56, and 69 of the DPbeta chain are involved in genetic susceptibility to autoimmune disease. For instance, the change at position 55 from alanine to aspartate occurs in both the DPB2.1 and DPB4.2 alleles; however, in IDDM, which appears to be linked to DPB2.1, there is also a change in the amino acid at position 69. It should be noted, though, that it is the entire allele, and not simply an amino acid at one position in an allele, which appears to confer susceptibility.



The effect of the HLA DP alleles on autoimmune disease susceptibility reflects similar findings in the HLA DR and HLA DQ loci. For instance, Scharf et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:3504-3508, and Horn et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:6012-6016, report that polymorphic residues at position 57 of the DQbeta chain and position 70 of the DRbeta 1 chain are implicated in genetic susceptibility to the autoimmune diseases pemphigus vulgaris (PV) and IDDM.

As noted above, it is anticipated that as medical technology develops, more disease or disease-prone states, including Grave's disease, systemic lupus erythematosus, and Sjögren's Syndrome, will become known to be associated with various DP alleles. The present invention provides methods for distinguishing such alleles from other alleles and so provides a means to identify individuals at high risk for an autoimmune disease. In a preferred embodiment, an individual whose susceptibility is to be determined is analyzed for HLA DP type first by using the PCR method to amplify the target region of the HLA DP locus. Then, SSO probes are hybridized to the amplified target region, and the particular DP allele present in the amplified DNA is determined from the pattern of binding of the SSO probes. Finally, one determines whether the allele present in the amplified DNA is an allele associated with the autoimmune disease.

The present method, however, is not limited to the field of medical science in ability to provide significant benefits. DNA typing methods also now play a significant role in the important area of individual identification, whether for solving crimes, as when the identity of a criminal or victim is established by linking an individual with evidence left at the scene of a crime, or for solving other issues of a non-criminal nature, as when biological material is used to determine the maternity or paternity of an individual.

Whatever the purpose for which the present invention is employed, the differences between various DP alleles is key to the success of the method. The most significant differences between DP alleles can be detected quite readily when the various amino acid sequences encoded by the alleles are aligned and examined. Such an alignment is shown below, where a dash indicates identity with the DPB4.1 allele (for the various DPbeta alleles and the DPbeta pseudogene, designated SXB) or with the DPA1 allele (for the DPalpha DPA2 allele and the DPalpha pseudogene, designated SXA). In this depiction, the numbered positions are for the mature peptide subunits, allele designations are at left, and representative cell sources are at right.

DPalpha Alleles

DP1: 10 20 30 40 50 60 70 80  
 DHVSTYAAVQTHRPTGEFMEFEDEMYVDLKKETVWHLEFGQAFSFEAQGLANIALNNNLTLQRSNHTQATN (LB)  
 DP2: 10 20 30 40 50 60 70 80  
 E S Y E Q N E M P P IHT D G R I G V MARK R NG KQ W D A (Daudi)  
 (SXA):

DPbeta Alleles

DPB4.1 10 20 30 40 50 60 70 80 90  
 NYLFCGRQECYAFNGTQRFLERYIYNREEFARFDSVCEFRVTELGRPAEYWNQSDILEEKRAVPDRMCRHNYELGPGHTLQRR (HHK)  
 DPB4.2 10 20 30 40 50 60 70 80 90  
 V V DE DE E E DEAP (APD)  
 DPB2.1 10 20 30 40 50 60 70 80 90  
 LV V E E DEAP (LB)  
 DPB2.2 10 20 30 40 50 60 70 80 90  
 V V DE DE E DEAP (OBL)  
 DPB8 10 20 30 40 50 60 70 80 90  
 LV V E E DEAP (Plaz)  
 DPB5 10 20 30 40 50 60 70 80 90  
 V V DE DE E DEAP (HAS)  
 DPB7 10 20 30 40 50 60 70 80 90  
 V V DE DE E DEAP (Raji)  
 DPB3 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (SLE)  
 DPB6 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (JMOS)  
 DPB11 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (CRK)  
 DPB13 10 20 30 40 50 60 70 80 90  
 Q Y Y X E I DEAP (BH)  
 DPB1 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (LUX)  
 DPB10 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (BM21)  
 DPB9 10 20 30 40 50 60 70 80 90  
 S VY L V DE DED L E DEAP (TOR)  
 DPB12 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (AKIBA)  
 DPB14 10 20 30 40 50 60 70 80 90  
 Q Y V V DE DED L E DEAP (8268)  
 DPB15 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (PLH)  
 DPB16 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (JRA)  
 DPB17 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (JRA)  
 DPB18 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (JRA)  
 DPB19 10 20 30 40 50 60 70 80 90  
 VY L V DE DED L E DEAP (JCA)  
 DPB20 10 20 30 40 50 60 70 80 90  
 SVY E W D G L Y V H A L M I G F F M R E V K V K ME LIR (Priess)  
 SXB

5 To detect and distinguish between DP alleles in a practicable and economic fashion, however, one must know the nucleotide sequence of the alleles. Portions of the nucleotide sequences of various DPalpha and DPbeta alleles are shown below; the sequences are identified as above. The illustrative primers of the invention enable production of DNA from which the sequence of codons 8 to 90 can be determined. The location of allele sequences that are the preferred target sequences for hybridization with the various probes of the invention are designated as [-A-], [-B-], [-C-], [-D-], [-E-], and [-F-].

	+10	+15	+20	+25
AsnTyrLeuPheGlnGlyArgGlnGluCysTyrAlaPheAsnGlyThrGlnArgPheLeuGluArgTyr				
AGAATTACCTTTCCAGGACGGCAGGAAATGCTACCGCTTTAATGGGACACAGCGCTTCCTGGNGATAC				
DPB4.1:				
DPB4.2:				
DPB2.1:				
DPB2.2:				
DPB8:				
DPB5:				
DPB7:				
DPB3:				
DPB6:				
DPB11:				
DPB12:				
DPB13:				
DPB1:				
DPB9:				
DPB10:				
DPB14:				
DPB15:				
DPB16:				
DPB17:				
DPB18:				
DPB19:				
DPB20:				

-----A-----|

	+30	+35	+40	+45	+50
DPB4.1:	IleTyrAsnArgGluGluPheAlaArgPheAspSerAspValGlyGluPheArgAlaValThrGluLeu				
DPB4.2:	ATCTACAACCGGGAGGAGTTCGGCGCTTCGACAGCGACGTGGGGGAGTTCCGGGGCGGTGACGGAGCTG				
DPB2.1:	-----T-----	-----T-----			
DPB2.2:	-----C-----	-----T-----			
DPB8:	-----T-----	-----T-----			
DPB5:	-----C-----	-----T-----			
DPB7:	-----T-----	-----T-----			
DPB3:	-----T-----	-----T-----			
DPB6:	-----T-----	-----T-----			
DPB11:	-----C-----	-----A-----	-----A-----		
DPB12:	-----T-----	-----T-----			
DPB13:	-----A-----	-----A-----	-----A-----		
DPB1:	-----A-----	-----A-----			
DPB9:	-----T-----	-----T-----			
DPB10:	-----T-----	-----T-----			
DPB14:	-----T-----	-----T-----			
DPB15:	-----C-----	-----A-----	-----A-----		
DPB16:	-----T-----	-----T-----			
DPB17:	-----T-----	-----T-----			
DPB18:	-----T-----	-----T-----			
DPB19:	-----T-----	-----T-----			

	+55	+60	+65	+70
	GlyArgProAlaAlaGluTyrTrpAsnSerGlnLysAspIleLeuGluGluLysArgAlaValPro			
	GGCGGCCTGCTGCGGAGTACTGGAACAGCCAGACATCTCGAGGAGAGCGGGCAGTGCCG			
DPB4.1:	---	---	---	---
DPB4.2:	A--A	---	---	---
DPB2.1:	A--A	---	---	---
DPB2.2:	AG	---	---	G
DPB8:	---	A--A	---	G
DPB5:	---	AG	---	G
DPB7:	---	---	---	---
DPB3:	A--A--C	---	---	---
DPB6:	A--A--C	---	C	---
DPB11:	A--A--C	---	C	G
DPB12:	A--A--C	---	C	---
DPB13:	---	---	---	G
DPB1:	---	---	---	G
DPB9:	A--A--C	---	---	---
DPB10:	A--A--C	---	---	G
DPB14:	A--A--C	---	---	G
DPB15:	---	---	C	---
DPB16:	A--A	---	C	G
DPB17:	A--A--C	---	---	G
DPB18:	A--A	---	---	G
DPB19:	AG	---	---	---
DPB20:	A--A--C	---	C	G

-----|-----D-----|

|---C---|

	+75	+80	+85	+90
AspArgMetCysArgHisAsnTyrGluLeuGlyProMetThrLeuGlnArgArg				
GACAGGATGTGCAGACACAACTACGACTGGGGGGCCCATGACCCCTGCAGGCCCGAG				
DPB4.1:				
DPB4.2:				
DPB2.1:				
DPB2.2:				
DPB8:	G-A		A-A-G-G	
DPB5:			A-A-G-G	
DPB7:	G-A		A-A-G-G	
DPB3:	G-A		A-A-G-G	
DPB6:			A-A-G-G	
DPB11:			A-A-G-G	
DPB12:	G-A	G	A-A-G-G	
DPB13:	A		A-A-G-G	
DPB1:	G-A		A-A-G-G	
DPB9:	G-A		A-A-G-G	
DPB10:	G-A		A-A-G-G	
DPB14:	G-A		A-A-G-G	
DPB15:			T	
DPB16:			A-A-G-G	
DPB17:			A-A-G-G	
DPB18:			T	
DPB19:	A		A-A-G-G	
DPB20:			A-A-G-G	

I-E-I

I---F---I

The DNA sequences provided above are an important aspect of the present invention. Although only one strand of the sequence is shown, those of skill in the art recognize that the other strand of the sequence can be inferred from the information depicted above. This information enables the construction of the probes of the invention. Many illustrative probes of the invention are shown in the Examples below. However, suitable SSO probes for hybridization analysis of the DPbeta alleles will comprise (or be complementary to) certain polymorphic sequences. Six sets of illustrative probes of the invention are depicted below; each set is designed to distinguish between the polymorphisms in a specific segment of the second exon of the HLA DPbeta gene. The designation of the segments is as described above. The polymorphic residues encoded within the allelic variant in the segment to which a probe hybridizes is shown in one letter amino acid code (the dash means that prototypic residues are present in those positions) to the left of the probe sequence. The probes span the regions encoding the polymorphic amino acid residues and are shown as having a length of about 18 nucleotides. Those sequences in the probe that encode the polymorphic amino acid residues, and thus must be included within a probe for detecting the alleles that encode the designated segment, are between the slash marks in the sequence. The DP alleles with which the probe will hybridize are shown to the right of the probe.



HLA DPbeta SSO Probes			
	Amino Acid	Probe Sequence	DPbeta Alleles
5	Segment A: LF-G:	TACCTTTTCCAGGGACGG	2.1, 2.2, 4.1, 4.2, 5, 7, 8, 19
	VY-L:	TACGTGTACCAAGTTACGG	3, 6, 11, 13
	VY-G:	TACGTGTACCAAGGGACGG	1, 15, 18
	VH-L:	TACGTGCACCAAGTTACGG	9, 10, 12, 14, 17, 20
10	Segment B: E-FA:	CGGAGAGGAGTTCCGCGCGC	4.1, 7
	E-FV:	CGGAGAGGAGTTCCGTGCGC	2.1, 3, 4.2, 6, 8, 9, 10, 12, 14, 16, 17, 18, 19, 20
	E-LV:	CGGAGAGGAGCTCGTGCGC	2.2, 5
	Q-YA:	CGGACAGGAGTACCGCGCGC	11, 15
	E-YA:	CGGAGAGGAGTACCGCGCGC	1, 13
	E-FV:	CGGAGAGGAATTCGTGCGC	10
15	Segment C: AAE:	CCTGCTGCGGAGTACTGG	1, 4.1, 7, 11, 13, 15
	DEE:	CCTGATGAGGAGTACTGG	2.1, 4.2, 8, 10, 16
	EAE:	CCTGAGGCGGAGTACTGG	2.2, 5, 19
	DED:	CCTGATGAGGACTACTGG	3, 6, 9, 12, 14, 17, 20
20	Segment D: I---K:	GACATCCTGGAGGAGAAG	1, 4.1, 4.2, 5, 7, 18
	I---E:	GACATCCTGGAGGAGGAG	2.1, 2.2, 8, 9, 10, 12, 13, 16, 17
	L---K:	GACCTCCTGGAGGAGAAG	3, 14, 20
	L---E:	GACCTCCTGGAGGAGGAG	6
	L---R:	GACCTCCTGGAGGAGAGG	11, 15
	Segment E: M:	GACAGGATGTGCAGACAC	2.1, 2.2, 4.1, 4.2, 5, 6, 11, 15, 16, 17, 18, 20
25	V:	GACAGGATGTGCAGACAC	1, 3, 7, 8, 9, 10, 12, 14
	Segment F: GGPM:	CTGGGCGGGCCCAATGACC	2.1, 2.2, 4.1, 4.2
	DEAV:	CTGGACGAGGCCGTGACC	1, 3, 5, 6, 7, 8, 9, 10, 11
	VGPM:	CTGGTCCGGGCCCAATGACC	15, 18

Because the probes of the invention are single stranded for use in hybridization, it is important to note that merely because a probe is designed to hybridize with, for example, the coding strand, does not mean that an equally useful probe could not be designed that would hybridize to the complementary sequence present on the noncoding strand.

The sequence information provided above also relates to other important aspects of the invention. The preferred primers of the invention are designed to amplify many different DP alleles. In many instances, as demonstrated in the Examples below, such primers are very useful. However, those of skill in the art recognize that the DNA

sequence information provided above can also be used to design primers that will enable allele specific amplification. Such allele-specific primers will only amplify a single allele or a certain subset of known alleles. For example, the "DEAV" probes of the invention can be used as one primer of a primer pair to provide for allele-specific amplification of "DEAV" DPbeta alleles.

The present invention also relates to kits that comprise a multicontainer unit comprising essential components for practicing the present method. For example, the kit can contain primers for PCR, as such primers are necessary in the preferred embodiment of the invention. These primers will amplify at least the DPbeta gene, and, when appropriate, for example in forensic analysis, primers can be included that also amplify the DPalpha gene. The kit must also contain SSO probes for at least the DPbeta gene, and, when appropriate, for the DPalpha gene as well. In some cases, the SSO probes may be fixed to an appropriate support membrane which is useful for the hybridization analysis. Other optional components that may be contained in containers within the kit include, for example, an agent to catalyze the synthesis of primer extension products, the substrate nucleotides, means used to label (for example, an avidin-enzyme conjugate and enzyme substrate and chromogen if the label is biotin), and the appropriate buffers for PCR or hybridization reactions. In addition to the above components, the kit can also contain instructions for carrying out the present method.

A number of examples of the present invention, which are provided only for illustrative purposes and not to limit the scope of the invention, are presented below. Numerous embodiments of the invention within the scope of the claims that follow the examples will be apparent to those of ordinary skill in the art from reading the foregoing text and following examples. In the following examples, certain techniques were standard, unless specifically indicated otherwise. Such techniques include PLT, which was performed essentially as described by Shaw et al., 1980, *J. Exp. Med.* 152:565-580. Generally, for lymphocyte priming, responder and stimulator cells were thawed, washed, and resuspended in RPMI-1640 medium supplemented with glutamine and antibiotics (complete media). Responding cells were mixed with irradiated stimulator cells in a ratio of 2:1, and the cell mixture was incubated for ten days at 37 degrees C. The primed irradiated stimulator cells were co-cultured with irradiated stimulator cells in complete media. After 48 hours, <sup>3</sup>H-thymidine was added to the culture. The cells were harvested 18 hours later, and tritium incorporation was evaluated by counting beta-emission.

DNA sequence analysis was performed as described by Maniatis *et al.*, Molecular Cloning: A Laboratory Manual (New York, Cold Spring Harbor Laboratory, 1982). Generally, the sequences to be analyzed were cloned into an M13 cloning vector and analyzed by either the Maxam-Gilbert technique or by the dideoxy chain termination technique. Synthetic oligonucleotides, both primers and probes, were synthesized using commercially available instruments and techniques well known in the art.

#### Example 1

##### Analysis of the DNA Sequences of HLA DP Alleles

The DNA sequences of the variable second exons of a variety of alleles of the DPalpha gene and of the DPbeta gene were determined. The DNA samples used were chosen to represent as wide a spectrum of PLT defined DP alleles as possible. DNA was extracted from cell lines homozygous for the standard six DPw types, from cells exhibiting unusual typing reactions, and from cells exhibiting DP blank reactions. DNA extraction was by standard techniques, as described by Maniatis *et al.*, in Molecular Cloning: A Laboratory Manual. The variable second exons of the DPalpha and the DPbeta genes were amplified by the PCR method, as described. The amplified DNA sequences were cloned into an M13 derived vector, and the DNA sequences were determined by the chain termination method. The cell lines used, their DR serotypes, their PLT defined DPw types, and the DNA defined alleles they were found to contain are listed in tabular form below (blank spaces indicate data not determined).

30

	Cell Line	DR Type	DPw Type	DPB Alleles	DPA Alleles
5	CRK	7	1*	1, 11	
	RSO101	w6	1, 3	1, 3	
	BMG	4	1, 4	4.1	
	QBL	3	2	2.2	1
	WJR	2	2	2.1	
10	PJM	3	2, 3	2.1, 3	
	RMD	5, w13	2, 4	2.1, 4.1	
	JMOS	4, 5	2, 6	2.1, 6	
	SLE	w13	3	3	1
	COX	3	3	3	1
15	OPR	w8, w10	3, 4	3, 4.1	
	JAH	4	3, 4	3, 4.1	
	MRI	1, 2	3, 5	3, 5	
	HHK	w6	4	4.1	
	LB1	3, 7	4, MAS	1, 4, 4.2	
20	APD	w6	4*	2, 4	
	LUY	w8	1, 4	1, 4.1	1, 2
	WDV	w6	2, 4	2.1, 4.1	
	SMF	2, w12	4, 5	4.1, 5	
	BCR	4, w6	4, 6	4.1, 6	
25	GTER	w8, 9	5	5	
	HAS	4	5	5, 19	
	DKY	9	5	2.1	1
	BIN40	4	3, 6	3, 6	1
	LG2	1	?	4.1	
30	PIAZ	2.7		8	
	TOK	2		9	2
	BM21	w11		10	
	VLA	2, 3		11	
	GBA	1, 7		11	
35	CD1	3, 4	3, 6	10	
	CD2	7, (5)	2, 4	4.2, 10	
	CD8	3, 7	4	4.1, 4.2	
	CD11		2	2.1, 4.2	

DP "MAS" is provisional designation for a newly defined DP specificity related to the DPB4.2 allele. "CD" cell lines actually are samples from CD patients.

\* - refers to cells with unusual DPw phenotypes.

As shown from the foregoing, the DNA analysis of the HLA DP subtypes shows that specific sequences correlate with the known PLT-defined DPw typings, indicating that the polymorphic epitopes recognized by the primed T cells are on the DPbeta chain. For some DP types, e.g., DPw2 and DPw4, sequence analysis has revealed subtype variants. The variants for DPw2 have been designated DPB2.1 and DPB2.2; cells typed for PLT as DPw4 "new" (e.g., LB1) or DPw4\* (e.g., APD) contain the more rare DPB4.2 subtype. The DPB4.2 subtype is more related by sequence to the DPB2.1 allele than to the DPB4.1 allele. Individual CD11 is PLT typed as DPw2, but contains the closely related DPB2.1 and DPB4.2 alleles.

The results above also show that unique DPbeta sequences correspond to the DPw1, DPw3, DPw5, and DPw6 specificities, and these alleles have been designated to reflect this correlation. A few exceptions are, however, that cell line DKY has been typed as DPw5, but contains the DPB2.1 allele, and that individual CD2 is PLT typed as DPw2, but contains the DPB4.2 and DPB10 alleles.

### Example 2

#### PCR Amplification of the DPalpha and DPbeta Genes

The DPalpha and DPbeta genes of some of the cells described in Example 1 were amplified by PCR. The primers used, which were synthetic, are shown below. In this depiction, the left side primers, GH98 and DB01, are from the upper strand, and direct DNA polymerase to extend rightward. The right side primers, GH99 and DB03, are from the lower strand, and direct synthesis leftward. The areas of the genes to which the primers bind, and which will act as templates for primer extension are also shown. Lower case letters indicate bases in the primer that are not complementary to the target genomic DNA (shown in the opposite strand). These changes in the primers incorporate restriction enzyme sites (*Bam*HI or *Pst*I) at the ends of the amplified DNA and facilitate cloning of the amplified DNA. The oligonucleotide primers GH98 and GH99, which are used for the amplification of the second exon of DPalpha, amplify a 243 bp segment. The first two bp of the PCR product are from the intervening sequence which flanks the exon. The oligonucleotide primers DB01 and DB03 amplify a 294 bp segment of the second exon of DPbeta. The left 13 bp and the right 17 bp of the product are from the intervening sequence.

DEalpha Primers

----- GH98 -----> Phe Val Gln Thr His Arg Pro Thr ... Leu Asn Asn Asn Leu Asn Thr Leu Ile  
 CCGGACCGTGTCACTTATGCGCG TTT GTA CAG ACG CAT AGA CCA ACA ... TTG AAC AAC AAC TTG AAT ACC TTG ATC  
 TCGCTGTACACAGTTGATACGCGC AAA CAT GTC TGC GTA TCT GGT TGT ... AAC TTG TTG TTG AAC TTA TGG AAC TAG  
 CAGCGTTCCACCCACTCAGGCCAC  
 GTCCAGGTTGTGTGACgtCGTc  
 <----- GH99 -----

DEbeta Primers

----- D801 -----> Leu Phe Gln Gly Arg Gln Gln Cys Tyr ... Gln Leu Gly Gly Pro Met Thr Leu Gln  
 CAGggatCCGACAGATTAC CTT TTC CAG CCA CCG CAG GAA TGC TAC ... CAG CTG GGC GGC CCC ATG ACC CTG CAG  
 CCGGAGCGCGCTCTTTATG GAA AAG GTC CCT GGC GTC CTT ACG ATG ... CTC GAC CCG CCG GCG TAC TGG GAC GTC  
 CCGGCTCCACTCACTgaCGGcctg  
 <----- D803 -----

Hybridization of the primers and the synthesis of the elongated primer containing products were essentially as described in EP 258,017 and in the protocols provided by the manufacturer, PECL, of the Thermal Cycler used to perform the PCR. Amplification was for 28 cycles; however, more, i.e., 35, cycles can on occasion yield better results.

Two other illustrative primers of the invention for amplifying the second exon of DPbeta alleles are designated UG19 and UG21. The sequences of these primers and the other illustrative DPbeta primers of the invention are shown below.

Designation	<u>DPbeta Primers</u> Sequence	Side
DB01	CAGGGATCCGCAGAGAATTAC	Left
10 DB03	GTCCTGCAGTCACTCACCTCGGCG	Right
UG19	GCTGCAGGAGAGTGGCGCCTCCGCTCAT	Left
UG21	CGGATCCGGCCCAAAGCCCTCACTC	Right

### Example 3

#### SSO Probes for Hybridization Analysis of DPbeta Alleles

Illustrative probes of the invention referred to throughout the remainder of the Examples are described below in tabular form. In the Table, the probe designation, probe sequence, polymorphic amino acid sequence encoded in the region of the allele to which the probe hybridizes, segment designation, and hybridization and wash conditions are shown. Probes are shown as having a  $^{32}\text{P}$  or "X" label, where X represents HRP, as discussed in the Examples below. Where a probe sequence is indicated by an X followed by a probe designation, the sequence of the probe is identical to that of the probe designated after the X, except that the  $^{32}\text{P}$  label has been replaced by an HRP label.

Those of skill in the art recognize that depending on the type of label used, hybridization and wash conditions will differ. Although, in a preferred embodiment, the probes will be labeled nonisotopically (e.g., with HRP), some of the probes have been used with isotopic (e.g.,  $^{32}\text{P}$ ) labels. Consequently, hybridization and wash conditions for  $^{32}\text{P}$ -labeled and HRP-labeled probes are shown, where such conditions have been empirically determined (see Bugawan et al., 1988, *J. Immunol.* 141(12):4024-4030). In the Table, the conditions referred to assume a hybridization solution composed of 5 x Denhardt's solution, 0.5% SDS, and the indicated amounts (i.e., 0.1 x, 3 x, 5 x) of SSPE. Five x Denhardt's solution contains 0.5 g Ficoll, 0.5 g polyvinylpyrrolidone, 0.5 g BSA (Pentax Fraction V) per 500 ml. The wash solution contains 0.1 x SSPE and 0.1% SDS

(for HRP-labeled probes, 0.1% Triton X-100 was used in place of SDS); the wash step is carried out at the indicated temperature for ten minutes. As described in Example 11, however, the use of tetramethyl ammonium chloride, or similar salts, can be used to allow for more uniform hybridization and wash conditions, a preferred condition when a number of probes are used in a panel to determine the types of DP alleles in a sample.



SSOs for HLA DPbeta Typing

	<u>Probe Designation</u>	<u>Amino Acid Sequence</u>	<u>Probe Sequence</u>	<u>Region</u>	<u>Hybridiz'n/Wash Conditions</u>
5	DB10	LFQG	32P-GAATTACCTTTTCCAGGGA	A	5 x @ 50°/42° H <sub>2</sub> O bath
	DB27	LFQG	X-DB10	A	5 x @ 50°/42° air
	DB11	VYQL	32P-ATTACGTGTACCAGTTACG	A	3 x @ 55°/42° H <sub>2</sub> O bath
	DB28	VYQL	X-DB11	A	3 x @ 55°/42° H <sub>2</sub> O bath
	DB58	VYQL	X-ATTACGTGTACCAGTTA	A	3-5 x @ 42°/42° air
10	DB23	VYQL	X-CGTAACCTGGTACACGTAAT	A	5 x @ 50°/42° H <sub>2</sub> O bath
	DB36	VYQL	X-DB23	A	5 x @ 50°/42° air
	DB12	VYQG	32P-CGTCCCTGGTACACGTAAT	A	5 x @ 50°/42° H <sub>2</sub> O bath
	DB29	VYQG	X-DB12	A	5 x @ 50°/42° H <sub>2</sub> O bath
	DB22	VHQL	32P-ATTACGTGCACCAGTTACG	A	3 x @ 55°/42° H <sub>2</sub> O bath
20	DB35	VHQL	X-DB22	A	3 x @ 50°/42° H <sub>2</sub> O bath
	DB13	AAE	32P-CCTGCTGCGGAGTACTG	C	3 x @ 55°/42° H <sub>2</sub> O bath
	DB30	AAE	X-DB13	C	3 x @ 50°/42° H <sub>2</sub> O bath
	DB14	DEE	32P-CAGTACTCCTCATCAGG	C	5 x @ 42°/42° H <sub>2</sub> O bath
	DB31	DEE	X-DB14	C	5 x @ 42°/42° H <sub>2</sub> O bath
25	DB16	EAF	32P-CAGTACTCCGCCTCAGG	C	5 x @ 42°/42° H <sub>2</sub> O bath
	DB32	EAE	X-DB16	C	3-5 x @ 42°/42° H <sub>2</sub> O bath
	DB59	EAE	X-CCTGAGGCGGAGTACTG	C	5 x @ 50°/42° H <sub>2</sub> O bath
	DB17	DED	32P-CCTGATGAGGACTACTG	C	5 x @ 50°/42° H <sub>2</sub> O bath
	DB33	DED	X-DB17	C	5 x @ 50°/42° air
30	DB18	I-K	32P-GACATCCTGGAGGAGAAGCD	D	0.1x @ 55°/42° H <sub>2</sub> O bath
	DB34	I-K	X-DB18	D	3 x @ 55°/42° H <sub>2</sub> O bath
	DB19	I-E	32P-GCTCCTCCTCCAGGATGTC	D	5 x @ 50°/42° H <sub>2</sub> O bath
	DB37	I-E	X-DB19	D	5 x @ 55°/42° H <sub>2</sub> O bath
	DB20	L-K	32P-GACCTCCTGGAGGAGAAGCD	D	3 x @ 55°/42° H <sub>2</sub> O bath
35	DB38	L-K	X-DB20	D	5 x @ 55°/42° H <sub>2</sub> O bath
	DB21	L-E	32P-GCTCCTCCTCCAGGAGGTC	D	5 x @ 50°/42° H <sub>2</sub> O bath
	DB39	L-E	X-DB21	D	5 x @ 50°/42° air
	DB62	L-E	X-GACCTCCTGGAGGAGGAG	D	3 x @ 50°/42° H <sub>2</sub> O bath
	DB63	L-R	X-GACCTCCTGGAGGAGAGG	D	3 x @ 50°/42° H <sub>2</sub> O bath
40	DB25	GGPM	32P-CTGCAGGGTCATGGGCC- CCCG	F	5 x @ 50°/42° H <sub>2</sub> O bath
	DB40	GGPM	X-DB25	F	3 x @ 50°/42° H <sub>2</sub> O bath
	DB26	DEAV	32P-CTGCAGGGTCACGGCCT- CGTC	F	5 x @ 50°/42° H <sub>2</sub> O bath
	DB41	DEAV	X-DB26	F	3 x @ 50°/42° H <sub>2</sub> O bath

With reference to the Table above, one should note that because DB28 cross-hybridizes with DB32, superior results can be obtained using probes DB58 and DB59 in place of DB28 and DB32, respectively. In addition, probe DB63 is preferred over DB39.

Example 4SSO Probes for Hybridization Analysis of DPalpha Alleles

Examples of suitable SSO probes for hybridization analysis of DPalpha alleles are shown below. Two sets of probes are illustrated; each set is designed to distinguish between the polymorphisms in a specific segment of the second exon of HLA DPalpha gene. ASO1 and ASO2 bind to the DPA1 allele at the region containing the polymorphic segments containing methionine (M) and glutamine (Q), respectively. ASO3 and ASO4 bind to the DPA2 allele to the region containing the polymorphic segments which contain glutamine and arginine (R), respectively. ASO2 and ASO4 distinguish the polymorphic segment containing glutamine from that containing arginine. The probes span the regions encoding these polymorphic amino acid residues. Hybridization using these probes is usually carried out in a solution containing 5 x SSPE, 5 x Denhardt's, and 0.5% SDS, for at least 1 hour at 42°C. Also shown are the washing conditions for use with the probes.

HLA DPalpha SSO Probes

Probe	Sequence	Washing Conditions
ASO1	AGATGAGATGTTCTATG	2 x SSPE, 0.1% SDS, 42°
ASO2	GTTTGGCCAAGCCTTTT	2 x SSPE, 0.1% SDS, 50°
ASO3	AGATGAGCAGTTCTATG	2 x SSPE, 0.1% SDS, 50°
ASO4	GTTTGGCCGAGCCTTTT	2 x SSPE, 0.1% SDS, 55°

Example 5Analysis of Amplified DPbeta Sequences by Hybridization with SSO Probes

PCR amplified DPbeta sequences from 24 HTC's were analyzed in a dot blot format with a panel (n=9) of <sup>32</sup>P-labeled SSO probes, and the DP type was inferred from the pattern of probe binding. The extraction of DNA from the cells was as described in Example 1. The target regions of the cellular genome, i.e., the second exon of the DPbeta genes, were amplified by the PCR technique as described in Example 2, except that the DNA was from the cells listed in tabular form above.

The amplified DNAs were dot blotted onto a filter; a separate filter containing the panel of samples was prepared for analysis by hybridization with each SSO probe. To dot blot the samples, five microliters of each amplified sample were diluted with 195 microliters of a solution containing 0.4 N NaOH and 25 mM EDTA and spotted onto 9

replicate Genatran 45 (Plasco) nylon filters by first wetting the filters with water, placing them in a Bio-dot (Bio-Rad, Richmond, CA) apparatus for preparing dot blots, applying the samples, and rinsing each well with 0.4 ml of 20 x SSPE (3.6 M NaCl, 200 mM  $\text{NaH}_2\text{PO}_4$ , and 20 mM EDTA). The filters were removed, rinsed in 2 x SSPE, and baked for 30 minutes at 80°C in a vacuum oven.

The samples on the filters were hybridized with SSO probes of the invention. Hybridization was with 0.25 to 0.5 pmoles of probe in 2 to 5 ml of hybridization solution. Hybridization and wash conditions were as described in tabular form in Example 3. The results of this DPbeta typing are shown below, which also shows the probe with which the samples on the filter were hybridized and the encoded amino acid sequence detected by the probe.

## DPbeta Oligonucleotide Probes

CELL LINE	HLA DR	HLA DPw	A				C			D			DPbeta Allele type
			DP10 LFQG 4.2.5, 7.8	DB11 VYQL 6.3.11 1	DB12 VYQG 1	DB13 AAE 4.1.7, 1.11	DB14 DEE 4.2.8, 2.1.10	DB17 DED 6.3.9, 12	DB18 I-K 4.5.7, 1	DB19 I-E 2.8.9, 10.12	DB20 L-K 3		
*1 COX	3	3	-	+	-	-	-	+	-	-	+	3	
2 950	3	1	-	-	+	+	-	-	+	-	-	1	
*3 BM21	11	blank	-	+/+	-	-	+	-	-	+	-	10	
4 BM16	12	2	+	-	-	-	+	-	-	+	-	2.1 (or 8)	
5 BR1G	5	2	+	-	-	-	+	-	+	+	-	2.1/4.2 or 4.2/8	
6 JYM	6	2	+	-	-	-	+	-	-	+	-	2.1 (or 8)	
7 WT46	6	2	+	-	-	-	+	-	-	+	-	2.1 (or 8)	
8 MANN	7	2	+	-	-	-	+	-	-	+	-	2.1 (or 8)	
9 J640	4	3	-	+	-	-	-	+	-	+	-	3	
10 SLE	13	3	-	+	-	-	-	-	-	-	+	3	
11 JMF	7	4	-	+	-	-	-	-	-	-	+	3	
12 BGE	2	4	+	-	-	+	-	-	-	+	-	4.1 (or 7)	
13 BM14	4	4	+	-	-	+	-	-	+	+	-	4.1 (or 7)	
14 BM92	4	4	+	+/+	-	-	-	-	+	+	-	4.2	
15 TS10	4	4	+	-	-	+	-	-	-	-	-	4.1 (or 7)	
16 RLO	4	4	+	-	-	+	-	-	+	+	-	4.1 (or 7)	
*17 APD	6	4	+	-	-	-	+	-	+	+	-	4.2	
18 BUP	7	4	+	-	-	+	+	-	+	+	-	4.1/4.2	
19 MMR	4	4	+	-	-	+	-	-	+	+	-	4.1 (or 7)	
*20 LUY	8	4/1	+	-	-	+	-	-	+	+	-	1/4.1 (or 7)	
21 YOS	4	4	+	-	+	+	-	-	+	+	-	4.2	
*22 DKY	9	5	+	-	-	-	+	-	-	+/+	-	2.1 (or 8)	
23 HAS	4	5	+	-	-	-	-	+	-	+	-	5	
*24 TOK	2	blank	-	+/+	-	-	-	-	+	+/+	+	9	

\* Cell line DPbeta type confirmed by DNA sequencing. +/- designates weak signal. Superscript c indicates the presence of an additional polymorphic sequence in region A encoding the residues VHVL, which cross-hybridizes with the DB11 probe (no cross-hybridization occurs with probe DB22). Superscript d represents cross-hybridizing due to the ability of the DB11 probe to bind to sequences recognized by the DB10 probe (cell line BM92) or due to the ability of the DB19 probe to bind to sequences recognized by the DB18 probe (cell lines YOS and HAS).

The determination of DPbeta types of the cells based upon the hybridization analysis with the SSO probes has been discussed above. To determine the DPbeta type of a sample, the binding of the probes to the sample was examined. The alleles present were inferred from the pattern of probe binding. For example, sample 1 formed hybrids with SSO probes DB11, DB17, and DB20. The amino acids encoded by DB11, DB17, and DB20 are VYQL, DED, and LEEK, respectively. An examination of segments A, C, and D of the DPbeta allelic amino acid sequences shows that the sequence VYQL is present in DPB6, DPB11, and DPB13; the sequence DED is present in DPB17, DPB14, DPB12, DPB9, DPB6, and DPB3; the sequence LEEK (L\_\_\_\_K) is present in DPB18, DPB14, DPB4.1, DPB4.2, DPB5, DPB7, DPB3, and DPB1. The only allele which contains the three sequences which hybridize with the probe is DPB3. Thus, the DP type of sample 1 based on SSO typing is DPB3.

The DPB types of the other samples were inferred by the same type of analysis, and the determined types are depicted above. In the depiction, the asterisk (\*) indicates cells where the DPbeta genotype was also determined by sequence analysis. The DPw type of the cell lines COX (formerly w1→w3) and BM21 (formerly w1→blank) have recently been changed to the type indicated. The symbol ± refers to a weak signal obtained with a probe. In some cases (BM21 and TOK), this weak signal reflects the presence of an additional polymorphic sequence in region A encoding the amino acid residues VHYL, which cross-hybridizes to the DB11 probe. The sequence can be more conveniently typed with region A probe DB22. For other cell lines (BM92), there is apparently a background cross-hybridization of the DB11 probe to the sequences recognized by the DB10 probe. In similar fashion, cross-hybridization signals can occur with the DB19 probe on sequences complementary to the DB18 probe.

The panel of SSO used in this Example detects variation at only 3 of the 5 polymorphic regions and does not detect all allelic variants at these 3 regions. The typing system using the procedure of this example is simple and unequivocal for HTCs, but given the patchwork pattern of polymorphism, can occasionally give rise to ambiguous typing for heterozygous individuals if the hybridization pattern of the various probes can be interpreted as more than a unique pair of alleles. This ambiguity arises from the many different combinations of the DPbeta sequence variants that constitute the different DPbeta alleles. However, with the use of the additional SSO probes provided by the invention,

which additional probes span the remaining polymorphic regions, unambiguous typings can be obtained for heterozygous individuals.

#### Example 6

##### HLA DP Typing of Coeliac Disease Patients by DNA Sequence Analysis of PCR Amplified Target Regions

5 The cells of four patients with coeliac disease (CD) were PLT-typed, and the DNA sequences of the second exon of the DPbeta determined as described in Example 1. The CD diagnosis was based on clinical symptomology.

10 The results of the analysis obtained by DNA sequencing of the DPbeta (second exon) of the CD cells has been discussed above. From these results, one observes that, when CD cells are compared to non-CD cells, there is an apparent increase in the frequency of the DPB4.2 allele. In addition, the DPB10 allele sequence is present in two independent CD patients yet observed in only one of the 30 non-CD cell lines.

#### Example 7

##### HLA DP Typing of Coeliac Disease Patients by SSO Probe Hybridization Analysis

15 The cells of 19 patients with CD and of 43 non-CD controls were analyzed for HLA DP type by SSO probe hybridization analysis. The diagnosis of CD was based on clinical symptomology; the CD patients as well as control individuals were all from Italy. DNA  
20 extraction was as described in Example 1. PCR amplification of the samples was as described in Example 2. Analysis of the amplified sequences was as described in Example 4.

25 The results of the analysis showed that there was a significant increase in the DPB4.2 allele in CD patients as compared to non-CD controls; this allele was present in 12 of 19 CD patients, but only 3 of 43 control patients. The DPB4.2 and DPB3 alleles were present in 17 of 19 CD patients and in 15 of 43 controls. The genotype DPB4.1/4.2 was present in 10 of 19 CD patients and in only 1 of 43 controls.

#### Example 8

##### HLA DP Typing of Forensic Samples

30 Samples which contain genomic nucleic acids of the suspect individual are obtained. The target regions of the genome, i.e., the regions containing the second exon of

the DPalpha and DPbeta genes, are amplified by the PCR technique, as described in Example 2, except that the nucleic acids from the suspect replace those of the cells in the Example, and except that the amplified samples contain a  $^{32}\text{P}$ -label. The amplified sample is hybridized with the probes of the invention, which have been dot blotted onto the same filter, and which have been immobilized on the filter by poly-dT tails. The hybridization and washing conditions for the filter allow only perfectly matched hybrids to remain in duplex state. The filters are examined to determine which probes form hybrids with the labeled sample.

A sample which is to be compared with that obtained from the suspect is examined for DP type by the same procedure, i.e., by PCR amplification and hybridization with immobilized SSO probes. The pattern of binding to the SSO probes of the sample from the suspect and of the comparison sample is examined to determine the similarity or difference in the hybridization patterns.

#### Example 9

##### HLA DP Typing Using SSO Probes Labeled with Horseradish Peroxidase

A panel of cells from 39 individuals was typed for DPbeta alleles using SSO probes for the second exon variants. The probes were labeled with horseradish peroxidase (HRP), and hybrids detected in a dot blot format.

The cells analyzed were from fourteen IDDM patients, five DR3 control non-IDDM patients, and nineteen HTCs which typed by PLT as DP blank. IDDM patients were identified by clinical symptomology. DNA was isolated from the cells as described in Example 1. The target region, i.e., the second exon of the DPbeta alleles, was amplified using the PCR technique in 200 microliters of reaction mixture that contained 50 mM Tris-HCl, pH 8.3; 2.5 mM  $\text{MgCl}_2$ ; 100 micrograms/ml gelatin; 0.75 mM in each of the four deoxynucleoside triphosphates; the primers DB01 and DB03; and Taq polymerase. The ramping for amplification was the following: heating for 30 seconds to  $94^\circ\text{C}$  followed by incubation at that temperature for 30 seconds; cooling for 1 minute to  $55^\circ\text{C}$  followed by incubation at that temperature for 30 seconds; heating to  $72^\circ\text{C}$  for 30 seconds followed by incubation at that temperature for 45 seconds. This cycling was repeated for 42 cycles. After amplification, the reaction mixtures were sampled and monitored by gel electrophoresis on gels containing 3% Nusieve, 1% Agarose to determine if all the DNA amounts were comparable.

Filters containing the dot blotted samples were prepared by blotting 150 microliters/dot of denatured amplified DNA on Genatran nylon membranes and UV treating the filters containing the samples for 5 minutes. The latter treatment is to fix the sample to the membranes. The amplified DNAs were denatured by treating 5 microliters of the PCR reaction mixture in a total volume of 150 microliters containing 0.4 N NaOH and 25 mM EDTA. Eight replicate filters were prepared for hybridization with HRP labeled ASO probes.

Prior to hybridization, the sample containing filters were incubated for 15 minutes in pre-hybridization solution (1 x SSPE, 5 x Denhardt's solution, 1% Triton X-100) without probe. In the pre-hybridization solution, Triton X-100 was used in place of SDS. Hybridization was in the same solution, which additionally contained 1 picomole probe/ml. Each filter was incubated for 40 minutes with 2.5 ml of hybridization solution containing one of the HRP labeled probes. The probes used were DB27, DB28, DB29, DB30, DB31, DB32, DB33, and DB35. The probes and hybridization conditions are listed in tabular form in Example 3. After hybridization, the filters were washed as stated in Example 3, under suitably stringent conditions, i.e., in 0.1 x SSPE, 0.1% Triton X-100 for 5 minutes at 42°C. The HRP-labeled SSO probes were prepared essentially by the methods disclosed in PCT publications 89/02931 and 89/02932, incorporated herein by reference.

These methods essentially involve derivatizing the nucleic acid probe using a linear linking molecule comprising a hydrophilic polymer chain (e.g., polyoxyethylene) having a phosphoramidite moiety at one end and a protected sulfhydryl moiety at the other end. The phosphoramidite moiety couples to the nucleic acid probe by reactions well known in the art (e.g., Beaucage et al., 1988, *Tetrahedron Lett.* 22:1859-1862), while the deprotected sulfhydryl group can form disulfide or other covalent bonds with the protein, e.g., HRP. The HRP is conjugated to the linking molecule through an N-maleimido-6-aminocaproyl group. The label is prepared by esterifying N-maleimido-6-aminocaproic acid with sodium 4-hydroxy-3-nitrobenzene sulfonate in the presence of one equivalent of dicyclohexylcarbodiimide in dimethylformamide. After purification, the product is added to phosphate buffer containing HRP at a weight ratio of 8:1 HRP to ester. The oligonucleotide probe is synthesized in a DNA synthesizer, and the linking molecule having the structure  $(C_6H_5)_3CS-(CH_2CH_2O)_4-P(CH_2CH_2CN)[N(i-PR)_2]$  attached using phosphoramidite synthesis conditions. The trityl group is removed, and the HRP



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 89/02169

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>4</sup>: C 12 Q 1/68, C 07 H 21/04, // C 07 K 7/00

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>7</sup>

Classification System

Classification Symbols

IPC<sup>4</sup>

C 12 Q

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP, A, 0237362 (CETUS CORPORATION) 16 September 1987 see abstract; page 6; pages 23-26; pages 33-45	1
A	--	2-19
A	Proc. Natl. Acad. Sci. USA, volume 84, March 1987, J. J. Hyldig-Nielsen et al.: "Restriction fragment length polymorphism of the HLA-DP subregion and correlations to HLA-DP phenotypes", pages 1644-1648 see the whole article cited in the application.	1-19
A	Proc. Natl. Acad. Sci. USA, volume 85, January 1988, M.D. Howell et al.: "An extended HLA-D region haplotype associated with celiac disease", pages 222-226 see the whole article cited in the application	1-19

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## IV. CERTIFICATION

Date of the Actual Completion of the International Search

7th September 1989

Date of Mailing of this International Search Report

- 9. 10. 89

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T.K. WILLIS

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 109, no. 1, July 1988, (Columbus, Ohio, US), M. Maeda et al.: "HLA-DP typing by analysis of DNA restriction fragment length polymorphisms in the HLA-DP $\beta$ subregion", see page 175, abstract 1766k, & Hum. Immunol. 1988, 21(4), 239-48 --	1-19

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

US 8902169

SA 29070

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0237362	16-09-87	AU-A- 6996287	17-09-87
		JP-A- 62214355	21-09-87
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 89/02169

**I. CLASSIFICATION OF SUBJECT MATTER** (If several classification symbols apply, indicate all) <sup>6</sup>  
According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>4</sup>: C 12 Q 1/68, C 07 H 21/04, // C 07 K 7/00

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>7</sup>

Classification System

Classification Symbols

IPC<sup>4</sup>

C 12 Q

Documentation Searched other than Minimum Documentation  
to the extent that such Documents are included in the Fields Searched <sup>8</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP, A, 0237362 (CETUS CORPORATION) 16 September 1987 see abstract; page 6; pages 23-26; pages 33-45	1
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<sup>10</sup> Special categories of cited documents: 10

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involve an inventive step

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cannot be considered to involve an inventive step when the  
document is combined with one or more other such docu-  
ments, such combination being obvious to a person skilled  
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A	Chemical Abstracts, volume 109, no. 1, July 1988, (Columbus, Ohio, US), M. Maeda et al.: "HLA-DP typing by analysis of DNA restriction fragment length polymorphisms in the HLA-DP $\beta$ subregion", see page 175, abstract 1766k, & Hum. Immunol. 1988, 21(4), 239-48  --	1-19
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		JP-A- 62214355	21-09-87

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